



PHD

Interrelations of the complement system and immune complexes in Crohn's disease.

Thorp, C. M.

Award date:
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INTERRELATIONS OF THE COMPLEMENT SYSTEM

AND

IMMUNE COMPLEXES IN CROHN'S DISEASE

Submitted by

C.M. Thorp, B.Sc. (Hons.)

for the degree of Ph.D.

of the University of Bath

1979

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ACKNOWLEDGEMENTS

I would like to thank Professor R.J. Ancill for allowing me to work in his Department at the University of Bath and the Royal United Hospital and Dr. K.R. Gough for his co-operation in providing material from the patients with Crohn's disease in his care.

My thanks are also due to my supervisor, Dr. E.R. Richens, for her initiation of this project and her constant encouragement and support, and to Dr. N.D. Hall for all his help and useful advice during the course of this work and for allowing me to use the facilities at the Royal National Hospital for Rheumatic Diseases.

I am indebted to Mrs. Jackie Russell and Miss Vivian Winrow for their excellent technical assistance.

I must also thank Rachel who typed the manuscript with such patience and determination.

Finally, my thanks to all my friends who have encouraged me, tolerated me and bullied me during the writing of this thesis, especially Ian and most of all Ivan.

TO IVAN

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SUMMARY

The aim of this project was to demonstrate the presence of immune complexes (IC) in the sera of patients with Crohn's disease (CD). The integrity of the complement system (C') was also investigated and comparisons were made with similar investigations on patients with ankylosing spondylitis (AS) and rheumatoid arthritis (RA).

ICs were detected in the sera of 32.2% of patients with CD, 100% of sera from patients with AS and 55% of synovial fluids from patients with RA, as shown by assessment of anticomplementary activity (ACA). The method was believed to detect small ICs.

Although total haemolytic complement, as assessed by CH_{50} assay, was normal both C3 and C4 levels were consistently elevated in the sera of patients with CD. All these factors were depressed in patients with AS and RA.

Measurement of C' activity and serum factors C3 and C4 gives a static picture of the involvement of C' in CD. A more dynamic view could be obtained by the demonstration of C3 inactivation products in the sera of patients with CD. This was demonstrated in 32.9% of sera tested. This was taken as evidence of in vivo C' activation by ICs and it was suggested that elevated C3 and C4 levels were due to alterations in catabolism or synthesis.

No relationship was found between the presence of ICs and raised C' levels; these factors were not found to be related to duration of disease activity, steroid therapy or disease activity. Since no differences were observed between patients in active or quiescent phases of the disease, it was concluded that the disease is characterized by a continuing immunological process.

Gel filtration and immunoglobulin analysis of ACA positive sera revealed that ACA was confined to high molecular weight fractions containing IgG and IgA. It was concluded that ICs in the sera of patients with CD were composed of IgG and possibly IgA complexed with an unknown antigen.

ABBREVIATIONS

Abbreviations used in this thesis are as follows:

Ab	Antibody
ACA	Anticomplementary activity
ACTH	Adrenocorticotrophic hormone
ADCC	Antibody-dependant cell mediated cytotoxicity
Ag	Antigen
AS	Ankylosing spondylitis
AZA	Azathioprine
B-cell	Bursa-derived lymphocyte
C'	Complement system
\bar{C}	Activated complement enzyme
C1-C9	Complement components 1-9
C1-INH	C1 inhibitory factor
Clq	Complement component Clq
Clr	Complement component Clr
Cls	Complement component Cls
C3	Complement component C3
C3i	C3 inactivation products
C4	Complement component C4
CD	Crohn's disease
CFT	Complement fixation test
CH ₅₀	Unit of C' causing 50% lysis of sheep red blood cells
cm	centimeter
Con A	Concanavalin A
°C	Degrees celsius
EDTA	Ethylenediaminetetra-acetic acid
Factor B	C3 Proactivator (C3 ProA), Glycine rich β glyco-protein (GBG).

g	Gravitational force. All rates of centrifugation are quoted as average g values.
GI	Gastrointestinal
G-PC	Guinea-pig complement
IC	Immune complex
Ig	Immunoglobulin
KAF	Conglutinogen activating factor
Kg	Killogram
M	Molar
mM	Millimolar
mm	Millimetre
mg	Milligram
MHD	Minimum haemolytic dose
ml	Millilitre
MW	Molecular weight
nm	Nanometre
non-RA	Non-rheumatoid arthritis
OD	Optical density
p	Probability values
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PMN	Polymorphonuclear cell
Pred	Prednisolone
psi	Pounds per square inch
PWM	Pokeweed mitogen
RA	Rheumatoid arthritis
RES	Reticulo-endothelial system
s	Svedberg units
Sal	Salazopyrine
SD	Standard deviation

SF	Synovial fluid
SLE	Systemic lupus erythematosus
SRBC	Sheep red blood cells
TB	Tuberculosis
T-cell	Thymus-derived lymphocyte
t.d.s.	Three times daily
THC	Total haemolytic complement
UC	Ulcerative colitis
UV	Ultraviolet
μ l	Microlitres
χ^2	Chi squared
>	Greater than
<	Less than
%	Percent

CHAPTER I

GENERAL INTRODUCTION

1.1. DEFINITION AND DESCRIPTION OF CROHN'S DISEASE

Crohn's disease (CD) was first described by Crohn, Ginsburg and Oppenheimer (1932) as being distinct from other chronic inflammatory lesions of the bowel; CD had previously been confused with ulcerative colitis (UC), since they possess many common features, viz fever, diarrhoea and emaciation, leading eventually to obstruction of the small intestine. UC is usually confined to the colon. CD was originally thought to affect the terminal ileum only, but it has subsequently been confirmed that there may be involvement of all regions of the gastro-intestinal (GI) tract from the mouth to the anus. The colon is frequently affected. UC and CD are now distinguished by radiological examination after barium meal and by histological examination of biopsy specimens, though when CD is confined to the colon it can still be difficult to distinguish it from UC. CD may also manifest itself with extra-GI symptoms, such as uveitis, vasculitis, arthritis, liver disease and erythema nodosum, the significance of which will be discussed.

CD is then a chronic focal and frequently multiple inflammatory lesion of the alimentary tract, with a granulomatous histological appearance reminiscent of experimental delayed hypersensitivity at pathological sites.

The clinical features of the disease include ulceration of the mucosa accompanied by the typical transmural inflammation of the connective tissue. This process frequently leads to stenosis of the lumen of the intestine and the formation of localized fistulae. Fistulae may occur between adjacent loops of intestine, to the vagina and also from the perianal region to the outside of the abdomen. There may be a palpable mass, quite often in the right ileac fossa.

The chronic illness follows an unpredictable course, which varies with the individual, and consists of quiescent periods interspersed with debilitating exacerbations when the disease is at its most active.

The symptoms of CD include colicky pain, diarrhoea, nausea, especially after eating, severe weight loss, anaemia and malabsorption syndromes depending on which part of the gut is affected.

Pathologically the mucosa and submucosa have a characteristic "cobble-stone" appearance with gross thickening of the bowel wall due to oedema, inflammation and fibrosis. "Skip" lesions may occur, separated by apparently normal sections of intestine. Radiography after barium meal shows constrictions where the lesions occur. The characteristic histological lesion, seen in about 60% of cases bears a strong resemblance to that seen in sarcoidosis (Blackburn, Hadfield and Hunt, 1939). Both diseases feature non-caseating granulomata with multinucleate Langhan's type giant cells. This feature also suggests a parallel with intestinal tuberculosis (TB).

Some patients need no drug therapy and can be maintained on a careful diet, whereas others require high doses of steroids and immunosuppressants and eventually surgical resection of the diseased bowel. Drug treatment is essentially palliative, designed to control symptoms and avoid complications. Since the aetiology and pathogenesis of CD is largely unknown, specific treatment to effect a cure is not yet possible.

1.2. AETIOLOGY AND PATHOGENESIS

In the past many suggestions as to the cause of CD have been proposed. No single factor alone has been indentified which can produce chronic CD and it may well be a clinical syndrome produced by a variety of agents (Kyle, 1972). The granulomata suggest a mycobacterial infection despite the absence of caseation (Jewell and Hodgson, 1976). Tubercular enteritis commonly affects the terminal ileum and remains important in the differential diagnosis of CD, especially in those parts of the world where TB is still prevalent. The distinction may not be straight-forward, but can usually be made on the presence of miliary tubercles in other parts of the body, the nature of the granulomata and the identification of acid-fast bacteria. TB responds to antibiotics, whereas although CD can be benefited by antibiotic therapy, it usually responds favourably to steroid treatment. Tubercle bacilli have not been cultured from Crohn's tissue. Nevertheless it has been suggested that CD may follow intestinal TB infection. Feilding (1970) proposed that when resistance to *Mycobacterium tuberculosis* is low exposure to the organism causes classical TB but when resistance to *M. tuberculosis* is moderate or high the mycobacterium may become modified and when attached to a hapten, such as bile salts, may become antigenic in a different way. CD has been likened to sarcoidosis (Mitchell and Rees, 1971, Willoughby and Mitchell, 1971; Richens et al, 1973), because of the similarity of the granulomatous lesion and it has been suggested that moderate resistance to *M. tuberculosis* results in sarcoidosis with the lungs being the target organ, and when resistance is high CD may arise with the resulting pathological disorder being in the intestine (Feilding, 1970).

It has also been suggested that other bacteria may be involved (Jewell and Hodgson, 1976). *M. paratuberculosis* causes a chronic hyperplastic enteritis called Johne's disease in ruminants which most commonly affects the ileum, but other areas of the gut may be affected. A local reaction occurs whereby there is infiltration of lymphocytes and epithelioid cells but the mucosa does not become ulcerated and the bacilli are clearly seen in histological section. Despite the similarities, this organism has not been cultured from Crohn's tissue. Following reports that a typical mycobacterium infected with mycobacteriophages might be present in the tissues of patients with sarcoidosis, similar investigations were carried out on patients with CD but no such infection was demonstrated (Parent and Wilson, 1971).

Golde and McGill (1968) proposed that a mycobacterium, as yet unidentified, must be responsible for CD because of the efficacy of sulphonamides in some cases of CD.

Bacterial toxins produced in the lumen of the intestine have also been suggested to play a part in the aetiology of CD. Bacteria may interfere with normal catabolism and absorption of substances from the intestine (Kyle, 1972).

1.3. A TRANSMISSIBLE AGENT IN CROHN'S DISEASE

A transmissible agent in CD was first proposed by Mitchell and Rees (1970) after they demonstrated that granulomata could be produced in the footpads of mice after inoculation with material from diseased ileum and lymph nodes from patients with CD. Since then there have been many reports indicating that injections of crude or coarsely filtered homogenates of Crohn's tissue are capable of causing granulomata in the footpads of mice (Mitchell and Rees, 1971; Taub et al, 1974; Mitchell and Rees, 1976; Taub et al, 1976; Mitchell, Rees and Goswami, 1976) and in the intestine of rabbits (Cave et al, 1973; Cave, Mitchell and Brooke, 1975; Donnelly, Delaney and Healy, 1977), though other workers have failed to confirm this (Bolton et al, 1973; Heatley et al, 1975). Subsequent passage of granulomata from animal to animal (Mitchell and Rees, 1971; Cave, Mitchell and Brooke, 1975; Mitchell and Rees, 1976; Mitchell, Rees and Goswami, 1976) and the fact that the original homogenates retained the ability to produce granulomata after passage through 0.2 μ filters (Cave, Mitchell and Brooke, 1975; Mitchell and Rees, 1976; Mitchell, Rees and Goswami, 1976) indicated that the transmissible agent may be of viral origin. Recent improved techniques have shown viruses in Crohn's tissue. Farmer et al (1973) showed an increase in cytomegalovirus titres in UC and from one case of CD they isolated this virus. Aronson et al (1974) showed the presence of virus in Crohn's tissue and intestinal fluid of 10/18 patients by cytopathogenic effects in tissue culture. The significance of this in CD and other GI disorders remains controversial as similar effects have been seen in other diseases. However, particles with the properties of a picorna virus have been isolated and cultured from Crohn's tissue (Gitnick and Rosen, 1976; Gitnick et al, 1976; Geraint-James, 1978). Bacteria have been

identified in the submucosa of the intestine in patients with CD, by electron microscopy (Aluwihare, 1971; Parent and Mitchell, 1976). When the intestinal anatomy is altered by inflammation it is reasonable to expect viruses and bacteria to be present but whether they are of primary pathogenic importance remains to be seen. Epidemiological studies have failed to find evidence for time and space clustering in CD to support the concept of viral aetiology (Miller et al, 1976).

1.4. OTHER POSSIBLE CAUSES OF CROHN'S DISEASE (Kyle, 1972)

1. The earliest feature of CD is enlargement of regional lymph nodes and oedema leading to lymphatic obstruction. It has proved difficult to cause experimental ulceration by lymphatic blockage alone. (Reichert and Mathes, 1936; Sinaiko, 1946). Kalima and Collan (1970) showed that lymphatic obstruction in rats produced a transmural inflammation, but there were no granulomas.
2. Ingested foreign material has unconvincingly been suggested to be causative in CD on the basis of animal experiments. Instilling sand or talcum powder in the ileum of dogs has produced a CD effect with inflammation and giant cells (Chess et al, 1950). Similarly, carageenan when fed to rats can produce an UC-like effect (Marcus and Watt, 1969).
3. Heredity may be of some importance. Certain families show a tendency to develop inflammatory bowel disease, either CD or UC (Lennard-Jones, 1968).
4. Following trauma, antigenic material may be released into the circulation and since it is not normally present there it could initiate an immunological reaction (Edwards, 1969).
5. Psychological factors may play a role once the disease has been established, as this is a depressing disease (Edwards, 1969).
6. Cross reactivity and aetiological link with sarcoidosis, has been proposed (Phear, 1958, Williams, 1965; Mitchell and rees, 1971; Richens et al, 1973).

7. Immunological responses are thought to be important in the aetiology of CD because of the beneficial effects of immunosuppressants and corticosteroids (Willoughby et al, 1971).

1.5. IMMUNOPATHOLOGY OF CROHN'S DISEASE

There are large amounts of lymphoid tissue in the ileo-caecal region of the gut, such as the Peyer's patches and also in the mucosa. Clinical observations show that CD runs a chronic relapsing course, not unlike that of rheumatoid arthritis which is believed to be of immunological aetiology (Zvaiffler, 1974). Immunological memory is believed to be long and an abnormality of the immune response may account for the protracted history of CD. Various types of skin lesion may occur unpredictably. Erythema nodosum often occurs with CD and there may have been eczema earlier in life.

The humoral antibody (Ab) system seems to be unimpaired in C.D. Serum immunoglobulin G (IgG) appears to be normal but there may be elevated IgA levels, some of which is probably secretory IgA (Deodhar et al, 1969; Perrett et al, 1971). IgG turnover has been reported to be increased (Bendixen et al, 1968) and serum IgM levels are raised if abscesses are present (Jensen, 1970). The ability to mount primary and secondary responses to the bacteriophage ϕ x174 is reported to be normal (Bucknal et al, 1975).

There is however, much controversy over the integrity of the cellular immune response in inflammatory bowel disease. Depression of delayed hypersensitivity responses in the skin have been reported (Blackburn et al, 1939; Phear, 1958; Williams, 1965; Verrier-Jones et al, 1969). Other workers have disputed this (Binder et al, 1966; Fletcher and Hinton, 1967; Bolton et al, 1974).

In vitro hyporesponsiveness in lymphocyte transformation in patients with CD is well documented (Parent et al, 1971; Sachar et al, 1973; Richens et al, 1974; Meuwissen et al, 1975; Hodgson, Wands and Isselbacher, 1978). Walker and Greaves (1969) and Brown

et al (1970) reported reduced lymphocyte transformation in response to pokeweed mitogen (Brown et al, 1970). On this basis it was suggested that only certain populations of lymphocytes showed anergy. However, many other reports have shown normal lymphocyte transformation (Asquith et al, 1973; Ropke, 1972; Aas et al, 1972; Bolton et al, 1974; Bird and Britton, 1974). Corticosteroid therapy has been shown to reduce lymphocyte responsiveness to mitogens (Fauci and Dale, 1974; Ramer and Yu, 1978).

The discrepancies between various reports may in part be explained by the choice of controls and variations in methodology. In general it appears that there is impairment of cellular immunity in CD.

Analysis of sub-populations in circulating white cells in inflammatory bowel disease again gives contradictory results. One report suggested a slight decrease in the percentage of T-cells with normal or increased percentage of B-cells (Strickland et al, 1974) whilst another demonstrated that although the percentage of all lymphocytes was reduced, probably due to an increase of polymorphonuclear (PMN) cell numbers, the actual numbers of T- and B-cells were normal, though erythrocyte-anti-body-complement rosetting cells were reduced (Thayer et al, 1976).

Many studies have investigated the cell populations in the bowel mucosa in CD. This type of study is probably more relevant to the disease process. Overall increases in numbers of mucosal immunocytes have been reported (Strickland, 1975) in diseased areas of bowel sections from patients with CD. Strickland (1975) reports that increased numbers of B-lymphocytes are found in the lamina propria of diseased bowel and of these 60% are IgG staining,

whereas in control sections of bowel similar numbers of T- and B-cells were found and of the B-cells 85% were IgA staining and only 5% IgG staining. In the peripheral blood of patients with CD decreased numbers of T-cells were found (Strickland, 1975) suggesting a shift to the diseased intestine. Green and Fox (1975) also found a deficit of IgA containing plasma cells in diseased areas of bowel from patients with CD although overall numbers of plasma cells were increased in both involved and unaffected intestinal mucosa when compared to sections of bowel from control patients. Since locally produced IgA plays a dominant role in the maintenance of a barrier to antigenic material, possibly by reducing Ag adhesiveness to mucosal cells or by forming non-absorbable stable complexes with Ag, this barrier will be reduced, the permeability of the intestinal epithelium to antigenic material will be increased and may even be accentuated by the epithelial binding of IgG which is believed to inhibit IgA activity and even increase uptake of antigen (Ag) (Green and Fox, 1975). Locally increased permeability to Ag offers an explanation of many of the pathological features of CD. Electron microscopy (Aluwihare, 1971) has demonstrated clusters of different bacteria deep in intact intestinal mucosa of patients with CD. This indicates that entry of antigenic material into the gut wall may not simply be secondary to ulceration.

In contrast, other workers have failed to find any significant difference in lymphocyte counts of lamina propria and intestinal epithelium in patients with CD or in control subjects (Bird and Britton, 1974; Ferguson, Allan and Cooke, 1975). In addition, disease activity, steroid therapy and anatomical localisation of the disease or resection did not have a significant affect on

lymphocyte counts. Plasma cell counts, however, were found to be raised as reported by Green and Fox (1975). Again, disease activity, drug therapy and anatomical involvement did not make a significant difference in plasma cell counts. Animal experiments indicate that the presence of plasma cells in the lamina propria is secondary to antigenic stimulation (Ferguson, Allan and Cooke, 1975). It has been suggested that increased numbers of plasma cells could be there in response to an abnormal Ag present in the proximal intestine or to a substance not normally antigenic (Ferguson, Allan and Cooke, 1975).

1.6. IMMUNE COMPLEXES AND CROHN'S DISEASE

A further possible factor in the aetiology and pathogenesis of CD may be the formation and circulation of immune complexes (IC) in this condition. There is evidence for the presence of ICs in the sera of patients with CD (Doe, Booth and Brown, 1973; Jewell and MacLennan, 1973; Hodgson, Potter and Jewell, 1977a and 1977c; Teisberg and Gjone, 1975). It has been suggested that circulating ICs may be responsible for the extra-GI manifestations of the disease such as arthritis, uveitis, vasculitis, liver disease and erythema nodosum.

Two biological events may occur following the formation of ICs. Firstly in vitro investigations have shown that ICs may both induce and inhibit lymphocyte toxicity depending on culture conditions (Perlmann, Perlmann and Wigzell, 1972). Secondly, ICs are also known to induce activation of the C' system (Mayer, 1973). Activation of the C' system is relevant to the work described in this project.

1.6.1. THE COMPLEMENT SYSTEM

Briefly, the C' system consists of 11 serum proteins, denoted by C1-C9. The sequential interaction of the C' proteins results in a number of important biological consequences, most of which result from activation of the terminal components C3 to C9. Activation of C3 can be initiated by two discrete mechanisms known as the "classical" and the "alternative" pathways.

The sequence of events that follows activation of C' by both pathways are illustrated in Figure 1.1.

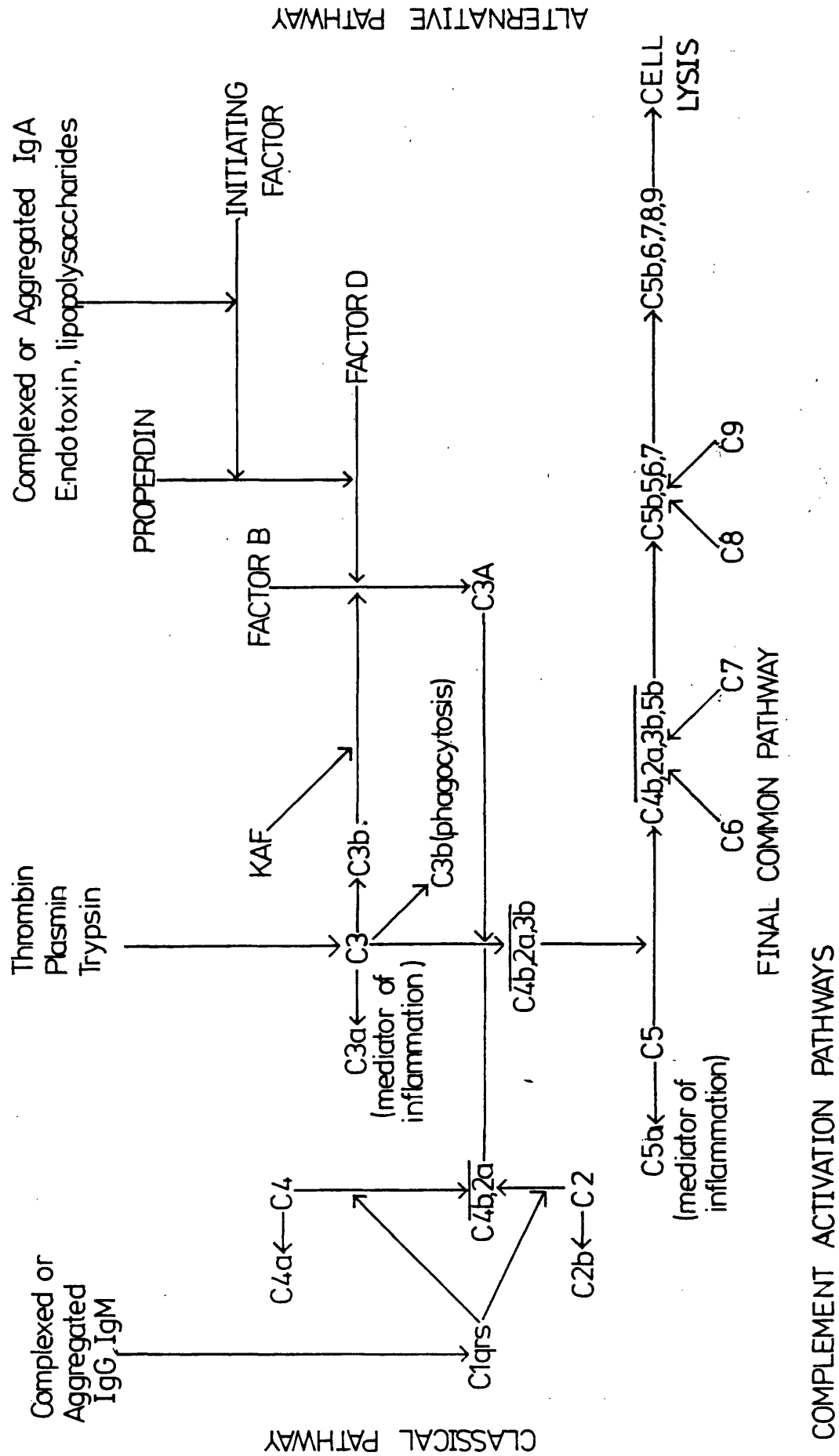


FIGURE 1-1

In the classical pathway, C1q is the recognition unit for the Fc portion of an Ab which becomes revealed or activated when it combines with its corresponding Ag. Only IgM and several subclasses of IgG are able to bind C1 in this way which results in its conversion to an active proteolytic enzyme with the active site being on the C1s sub-unit and the natural substrates being C4 and C2. Activated C1s is capable of cleaving many molecules of C4, resulting in a shower of C4b molecules, some of which become bound to the cell surface and others which quickly become inactivated and circulate in the blood. C2 is then adsorbed onto the bound C4b and is cleaved by C1s into C2a and C2b. C2a becomes bound to C4b and the resulting enzyme C4b2a is capable of cleaving many molecules of C3 resulting in a shower of C3a and C3b molecules. C3a is released into the fluid phase and plays a role as a mediator of inflammation. C3b becomes bound to receptors on the cell surface. A new enzyme is created, C4b2a3b, which is capable of cleaving C5 and so the sequence proceeds. C3b bound to other sites on the cell surface is believed to play a role in promoting phagocytosis.

In the alternative pathway, a system of serum proteins termed properdin is involved, which acting together with C', participate in several immunological reactions, including promotion of phagocytosis of cells and foreign particles and the production of inflammatory reactions. Activation of the alternative pathway is independent of C1, C2 and C4 and is initiated by polysaccharides from plant and bacterial cell walls and aggregated or complexed IgA. Factor B (synonyms C3 proactivator (C3 Pro A) and glycine rich β glycoprotein (GBG)) is the C2a counterpart and is activated by a protein, Factor D, to give C3 Activator (C3A) which is capable of activating C3. The reaction is then believed to proceed as in the classical

pathway. Although activation of the properdin pathway is said to be independant of C' components C1, C2 and C4, it is known that if C3b is present it catalyses the activation of C3A. This positive feedback loop is modulated by the C3 inactivator KAF (conglutinin-activating factor), which inactivates C3b. The two systems of C' activation are clearly interrelated (Takada, Imamura and Takada, 1979).

However C3 is activated, the sequence proceeds with release of C3a, an anaphylatoxin which can release histamine from mast cells and cause smooth muscle contraction, and from cleavage of C5 a similar small fragment C5a with histamine releasing activity and chemotactic properties for PMN leucocytes and monocytes. C5b forms a complex with C6 and C7 in the fluid phase and becomes bound to the cell membrane. Finally the terminal C' components C8 and C9 become bound and membrane damage with cell lysis results. C8 is believed to have a phospholipase-like activity and C9 is thought to enhance this activity.

1.7. IMPLICATIONS OF COMPLEMENT ACTIVATION IN CROHN'S DISEASE

The presence of ICs in serum is implicated by fixation and activation of C'. Sera that contain C'-fixing ICs will also fix C' in vivo. This project was designed to show the extent of C' activity and detection of ICs in the serum of patients with CD. In vivo C' activation was examined by measuring total haemolytic complement levels, serum concentration of C' components C3 and C4 and by demonstrating C3 inactivation products. The presence of ICs was determined by a method of measuring anticomplementary activity (ACA) in patients' sera. Sera that showed evidence of containing ICs were additionally analysed by gel chromatography in an attempt to localise the ACA to a particular molecular weight range and to characterise the immunoglobulin content of the ICs.

CHAPTER 2

MAT ERIALS AND METHODS

2.1. MATERIALS

All chemicals and reagents were of analytical grade whenever available and unless otherwise stated, came from normal commercial sources, such as British Drug Houses, Poole, Dorset. (Appendix II). The following specific materials were also used:

Sheep red blood cells (SRBC) in Alsevers solution (Oxoid);

Horse anti-SRBC antiserum (Wellcome);

Complement fixation test (CFT) buffer tablets (Oxoid);

0.04% ammonia solution;

Microtitre plates 8x12 U-shaped wells and lids (Titretek, Flow Labs.);

Tulip loop microdilutors, 25 ul. (Titretek, Flow Labs.);

25 ul. pipettes (Titretek, Flow Labs.);

Freeze-dried guinea-pig complement (GP-C) (Wellcome);

Ionagar No. 2 (Oxoid);

Barbitone buffer, pH 8.6, ionic strength 0.1 (Appendix I);

Antiserum to C3 and C4 (Hoechst);

Human serum standard (Hoechst);

Phosphate buffered saline (PBS) pH 7.35 ± 0.05 (Appendix I);

Coomassie Brilliant Blue (BDH) (Appendix I);

Hamilton microsyringe and adaptor (V.A. Howe);

Water baths at 56°C and 100°C;

Damp chamber at 4°C;

Well cutter (Wellcome);

Glass slides, 10x16.5 cm.;

Standard microscope slides, 1"x3"x1mm;

5ml. bijoux bottles;

Pasteur pipettes;

Spectrophotometer SP600 (Unicam).

2.1.1. BLOOD SAMPLES

For all the methods described in this Chapter blood samples were treated in the following manner:

Venous blood was collected into plain bottles and allowed to clot at room temperature. Within 2 hours of collection the serum was spun off and dispensed into 0.5 ml. aliquots and frozen immediately at -70°C . A fresh aliquot was thawed once only prior to use for each test.

2.2. METHODS

The methods of total haemolytic complement (Hudson and Hay, 1976) and anticomplementary activity (Johnson, Mowbray and Porter, 1975) assay and estimation of C3 and C4 concentrations (Mancini, 1965) are described as follows.

2.2.1. TOTAL HAEMOLYTIC COMPLEMENT

The principle of this assay is based on the fact that as complement (C') is added to antibody (Ab)-coated sheep red blood cells (SRBC), an increasing proportion of cells are lysed as shown in Figure 2.1. As it is difficult to determine the C' concentration that causes total lysis of the cells, the 50% lysis point is normally quoted, the CH_{50} .

The Von Krogh equation for the sigmoid dose-response curve of C'-mediated cytolysis was arrived at empirically (Hudson and Hay, 1976) and in its basic form is:

$$x = k(y/1-y)^{1/n}$$

where x = amount of C' (ml. of undiluted serum)

y = proportion of cells lysed

k = 50% unit of C'

n is a constant

The CH_{50} unit is an arbitrary unit dependent on SRBC and anti-SRBC concentration, buffering conditions of the medium, temperature, reaction time and concentration of calcium and magnesium ions. Standard conditions must therefore be carefully controlled.

The method used was a titrimetric assay, similar to the method described by Hudson and Hay (1976).

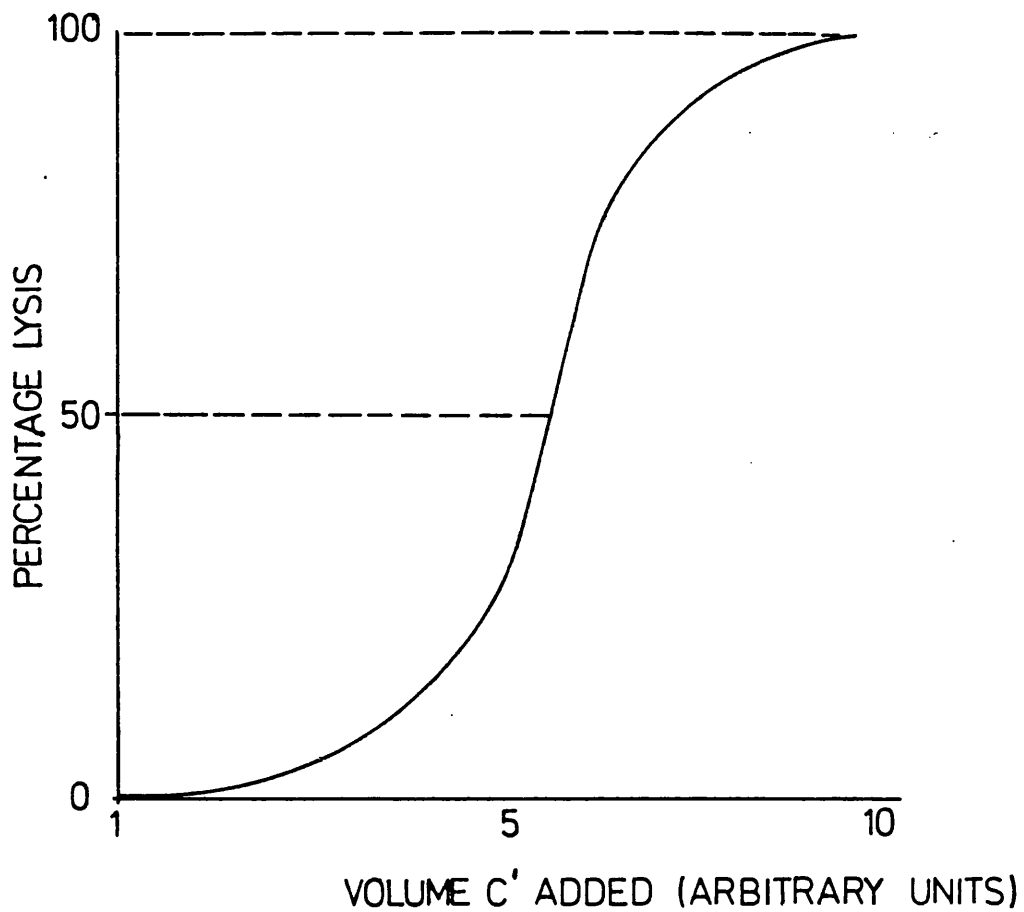


FIGURE 2.1

LYSIS OF SHEEP RED BLOOD CELLS (SRBC) BY HORSE ANTI-SRBC IN THE PRESENCE OF HUMAN COMPLEMENT (SERUM).

2.2.1.1. Method

5 ml. of SRBC were washed 3 times in CFT buffer and suspended in 15 ml. CFT buffer. 0.2 ml. of the suspension were added to 4.8 ml. 0.04% ammonia solution, this causing lysis of the cells. The optical density was read at 541 nm. A 6% suspension of SRBC gives an absorbance of 0.48-0.50 at 541 nm. The volume of the cells was adjusted until this concentration was obtained, according to the equation:

$$\text{volume} = \frac{15 \times \text{OD}}{0.49} \text{ ml}$$

0.1 ml. horse anti-SRBC antiserum was added to 15 ml. CFT buffer and to this was added 15 ml. of 6% SRBC suspension. The final suspension was incubated at 37°C for 15 minutes, thus giving a 3% suspension of sensitized SRBC.

Dilutions of the patients serum from 1/2 to 1/4096 were made with 25 µl. CFT buffer in the 12 horizontal wells of a microtitre plate using a tulip loop microdilutor. 25 µl. 3% sensitized SRBC suspension were titrated with the patients serum in a final volume of 100 µl. made up with CFT buffer. The haemolytic reaction was allowed to proceed for 60 minutes at 37°C with periodical shaking. The plates were left overnight at 4°C before reading. The unlysed cells settled out and formed a "button" in the bottom of the wells, the diameter of which depends on the volume of unlysed cells.

The titre of a patient's serum is the reciprocal of the highest dilution of serum giving 50% lysis of the sensitized SRBC, e.g. CH₅₀ 128.

2.2.1.2. Controls

On each plate one well was reserved for a control of 3 volumes of CFT buffer (75 μ l.) and 1 volume sensitized SRBC (25 μ l.).

2.2.2. ANTICOMPLEMENTARY ACTIVITY

The principle of this assay depends on the ability of immune complexes (IC) to bind C'. The patients serum is first incubated at 56°C for 60 minutes to inactivate intrinsic C' already bound to ICs, if they are present. Guinea-pig complement (GP-C) is then added to detect the degree of C' binding in a system of sensitized SRBC. The titrimetric method used was based on the method described by Johnson, Mowbray and Porter (1975).

2.2.2.1. Method

A 3% suspension of sensitized SRBC was prepared as described in the method of CH₅₀ assay.

The GP-C was first titrated with SRBC to give a concentration that would cause 100% lysis of SRBC before the patient's serum is added. The minimum haemolytic dose (MDH) of GP-C is the lowest dilution of GP-C that causes incomplete lysis of the SRBC. In the titration for ACA 2 MHD were used. After inactivation at 56°C for 60 minutes the patients serum was diluted from 1/2 to 1/4096 as described for CH₅₀ assay. To this was added 25 μ l. of 2 MHD of GP-C and 25 μ l. of CFT buffer. The C' fixation reaction was allowed to take place at 4°C overnight.

25 μ l. of sensitized SRBC were added and the haemolytic reaction allowed to proceed for 60 minutes at 37°C with periodical shaking. The plates were left overnight at 4°C before reading. The unlysed cells settled out to form a "button" in the bottom of the wells, the diameter of which depends on the volume of unlysed cells.

The titre of a patient's serum is the reciprocal of the highest dilution of serum, previously incubated overnight with 2MHD of GP-C, giving 50% lysis of the sensitized SRBC e.g. ACA 16.

2.2.2.2. Controls

Included in each plate was a row of diluted (1/2-1/4096) heat aggregated human immunoglobulin (IgG), original concentration 0.85 mg/ml.

One well was reserved for a control containing no GP-C or patient's serum, but 2 additional volumes of CFT buffer (50 μ l.).

A second well was reserved for a control containing GP-C but no patient's serum, and one additional volume of CFT buffer (25 μ l.).

2.2.3. C3 AND C4 CONCENTRATIONS

C3 and C4 concentrations were determined by single radial immunodiffusion (Mancini et al, 1965) using agar plates prepared in the laboratory.

2.2.3.1. Method

5 ml. of 1% agar made up in barbitone buffer, pH 8.6, ionic strength 0.1 were melted in a water bath at 100°C and transferred to a water bath at 56°C to equilibrate for 30 minutes. Using a Hamilton microsyringe, 50 μ l. of the appropriate antiserum (anti-C3 or anti-C4) was added to the molten agar and mixed by gentle rotation and inversion of the bijoux bottle.

3 microscope slides were assembled in a U-shape on one glass plate. A second glass plate was placed on top and the 2 glass plates were held together with the microscope slides sandwiched in between, by 3 bulldog clips.

Using a warmed Pasteur pipette, 5 ml. molten agar containing antiserum were introduced into the space formed by the microscope slides. The agar was allowed to solidify at room temperature, the plates being supported in vertical position. The top glass plate was carefully removed and the surface of the agar allowed to dry.

Using a template and gel cutter, 12 wells were cut in the gel.

Serum samples were diluted 1:1 with 0.9% saline and mixed gently. 1 μ l. and 2 μ l. of sample were applied to each well of the C3 plate and the C4 plate, respectively. There were 9 samples and 3 standards to each plate. Diffusion was allowed to take place over 72 hours in a damp chamber at 4°C.

Commercial human serum was used to make up 3 standards which were included in each plate.

The concentrations of the standards were:

C3; 22, 44 and 88 mg%

C4; 7.5, 15 and 50 mg%.

The plates were dehydrated by compression of the agar, covered with filter paper and absorbant tissue, with a 2 Kg. weight. After drying for 10 minutes at room temperature, the diffusion rings were visualised by staining in Coomassie Brilliant Blue for 5 minutes. Background stain was removed by washing in the destaining solution.

Diameter² (D^2) was plotted against concentration.

CHAPTER . 3

COMPLEMENT AND IMMUNE COMPLEX STUDIES ON PATIENTS
WITH CROHN'S DISEASE, ANKYLOSING SPONDYLITIS AND
RHEUMATOID ARTHRITIS AND CONTROL SUBJECTS.

3.1 INTRODUCTION

Many workers have reported evidence of immune complexes (IC) in the sera of patients with Crohn's disease (CD) using methods both based on the complement (C') system (Doe, Booth and Brown, 1973; Hodgson, Potter and Jewell, 1977a) and on the inhibition of antibody (Ab)-dependent cytotoxic reactions (Jewell and MacLennan, 1973). In addition, measurement of serum levels of various components of the C' system has revealed that they may be altered (Hodgson, Potter and Jewell, 1977b) or near normal levels (Teisberg and Baklien, 1974; and Teisberg and Gjone, 1975).

The involvement of ICs in the pathogenesis of rheumatoid arthritis (RA) and ankylosing spondylitis (AS) has been considered for many years (Norberg, 1974; Sturrock et al, 1975; Corrigal et al, 1978). ICs were first described in the sera of some patients with RA by Kunkel et al (1961), and later in synovial fluids (SF) by Winchester, Agnello and Kunkel (1970). Again, ICs in SF have been identified by methods utilizing activation of the C' sequence (Zvaiffler, 1974; Zubler et al, 1976) and by other methods (Barnett and MacLennan, 1972; Luthra et al, 1975). Alterations of C' levels in sera and SF have also been reported (Pekin and Zvaiffler, 1964; and Ruddy and Austen, 1970).

As reported levels of ICs and C' components in sera from patients with CD appeared inconsistent, it was decided to investigate some of these parameters in sera of patients with CD at the Royal United Hospital, Bath. The sera from these patients were compared with those of age and sex-matched normal healthy subjects. In addition, some positive controls were included, namely SF from patients with RA and

sera from patients with AS. A few SF from patients with non-rheumatoid arthritides (non-RA) were also included, i.e. monoarthritis, chondrocalcinosis and pseudo-arthritis.

3.2 PATIENTS AND METHODS

3.2.1 PATIENTS

The patients with CD were taken from in-patients and out-patients at the Royal United Hospital, Bath. The overall group included 69 patients, age range 18-76 years, mean age 40 years.

33 patients with AS were taken from in-patients and out-patients at the Royal National Hospital for Rheumatic Diseases, Bath. Their age range was 24-64 years, mean age 43.5 years.

20 patients with RA were taken from in-patients and out-patients at the Royal National Hospital for Rheumatic Diseases, Bath. Their age range was 18-69 years, mean age 49 years. 6 patients with non-RA were also taken from this hospital. Their ages were not known.

The group of 54 control subjects included laboratory personnel and student volunteers. They were age and sex-matched with the patient groups wherever possible, but the age range 18-60 years and mean age 27 years were lower than in the other groups.

As many sera and SF from patients and control subjects as possible were included in each series of determinations. Details of these are given in the results section.

3.2.2 METHODS

The methods of estimation of total haemolytic complement (THC), anti-complementary activity (ACA) and of C' components C3 and C4 have been described in Chapter II.

All blood samples were collected into plain bottles and allowed to clot at room temperature. Within two hours of collection the serum was centrifuged off, dispensed into 0.5 ml. aliquots and stored at -70°C.

SF were collected into heparin tubes and centrifuged at 600g for 20 minutes. The supernatant was dispensed into 0.5 ml. aliquots and stored immediately at -70°C . Before use, SF were incubated at 37°C for 15 minutes with 0.5 mg./ml. of hyaluronidase. This process reduced the viscosity of the sample to that of serum.

3.2.3. STATISTICS

The Kolmogorov-Smirnov (Seigel) two sample χ^2 test and students t-test were used to determine whether the CH_{50} and ACA results and the C3 and C4 estimations, respectively, differed in the patient groups from the values obtained for the control subjects.

3.3 RESULTS

The results of the various assays of sera and SF are presented as follows.

3.3.1. TOTAL HAEMOLYTIC COMPLEMENT

THC is expressed as a titre, this being the reciprocal of the highest dilution of sample giving 50% lysis of sensitized sheep erythrocytes, the CH_{50} . A low titre may indicate fixation of C' by ICs.

Details of patients, age range, mean age and number of samples tested are given in Table 3.1.

Figure 3.1 illustrates the comparative percentage distribution of CH_{50} titre. The normal range of CH_{50} titre, as shown by 89% of control subjects, is considered to be between 16 and 128. 88% of sera from patients with CD had activity within this range.

Statistical analysis of the cumulative percentage distribution revealed no significant difference between CH_{50} distribution in sera from control subjects and patients with CD ($\chi^2 = 3.264$, $p > 0.1$).

CH_{50} distribution was found to be significantly lower in sera from patients with AS and SF from patients with RA when compared to sera from control subjects ($\chi^2 = 74.41$, $p < 0.001$ and $\chi^2 = 13.7$, $p < 0.01$, respectively).

No significant difference was found between CH_{50} distribution in SF from patients with RA or non-RA ($\chi = 2.219$, $p > 0.2$). However, the group of patients with non-RA was probably too small for tests of statistical significance.

Patient Group	No. of Subjects	No. of Samples	Age Range (Years)	Mean Age (Years)
Crohn's Disease	60	116	18-76	40
Ankylosing Spondylitis	33	33	26-64	43.5
Rheumatoid Arthritis	20	20	18-69	49
Non-Rheumatoid Arthritis	5	5	NOT KNOWN	
Control Subjects	54	56	18-55	25.4

Table 3.1. CH₅₀ ASSAY

Details of patients and control subjects

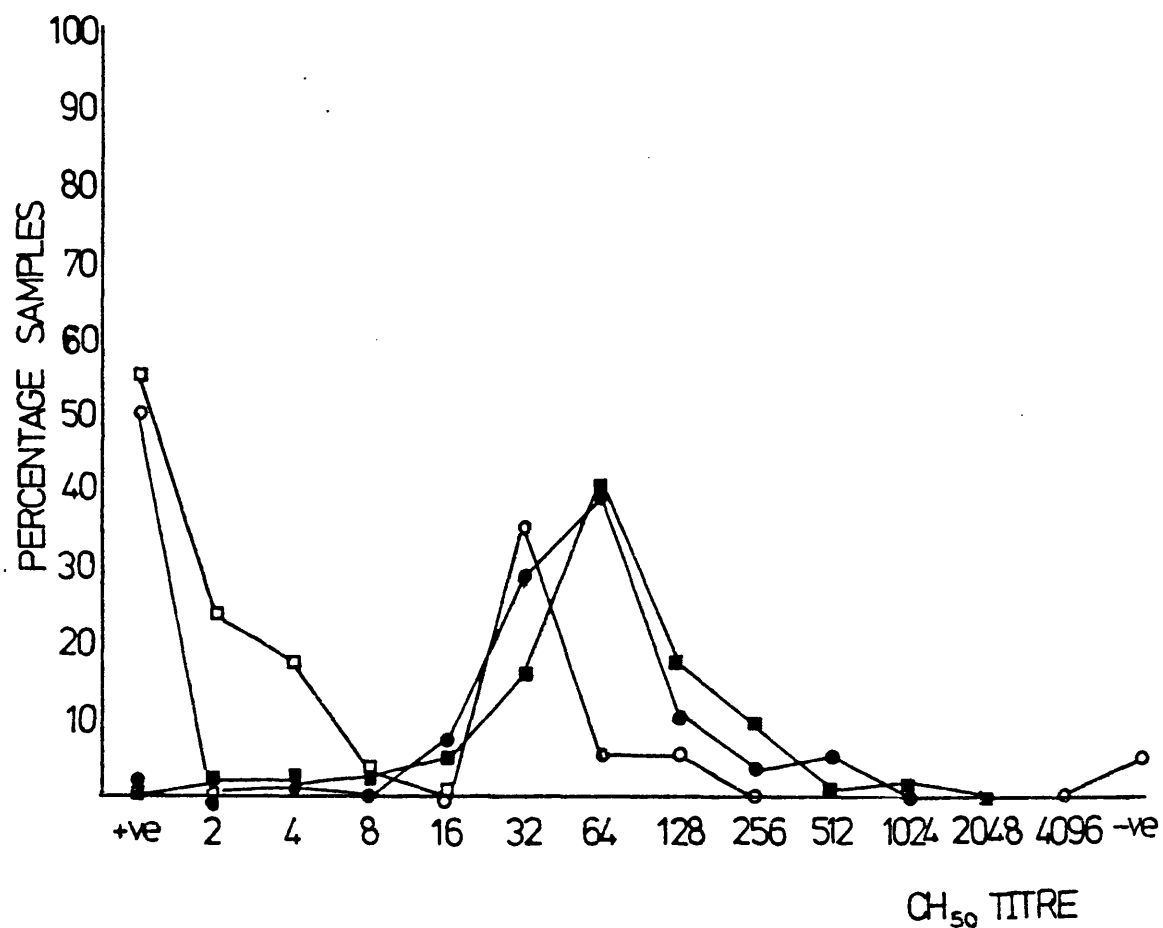


FIGURE 31

PERCENTAGE DISTRIBUTION OF CH₅₀ TITRE IN CONTROL SUBJECTS, (●-●), AND PATIENTS WITH CROHN'S DISEASE, (■-■), RHEUMATOID ARTHRITIS, (○-○), AND ANKYLOSING SPONDYLITIS (□-□).

3.3.2. ANTICOMPLEMENTARY ACTIVITY

ACA is expressed as a titre, this being the reciprocal of the highest dilution of sample, previously incubated overnight with two minimum haemolytic doses (MHD) of guinea-pig C' (GP-C), at which 50% lysis of sensitized sheep erythrocytes occurs. If total haemolysis occurs, it can be inferred that there are no ICs in the sample to cause fixation of endogenous C'. The higher the titre at which 50% lysis occurs, the higher the level of ICs in the sample. An extremely high content of ICs is indicated when no 50% end point can be discerned due to all the GP-C having been fixed.

Details of patients, age range, mean age and number of samples tested are given in Table 3.2.

Figure 3.2. illustrates the comparative percentage distribution of ACA titre.

96% of sera from control subjects had negative ACA and thus showed no evidence of ICs. 68.8% of sera from patients with CD also had negative ACA. Statistical analysis of the cumulative distributions revealed that sera from patients with CD had significantly raised ACA when compared to sera from control subjects ($\chi^2 = 10.41$, $p < 0.01$).

All of the sera from patients with AS showed ACA. The cumulative distribution was significantly higher than that from the control sera ($\chi^2 = 75.65$, $p < 0.001$).

45% of SF from patients with RA showed negative ACA, but the cumulative distribution was significantly higher than that of sera from control subjects ($\chi^2 = 15.03$, $p < 0.01$).

Patient Group	No. of Subjects	No. of Samples	Age Range (Years)	Mean Age (Years)
Crohn's Disease	55	107	18-76	39
Ankylosing Spondylitis	33	34	26-64	43.5
Rheumatoid Arthritis	20	20	18-69	49
Non-Rheumatoid Arthritis	5	5	NOT KNOWN	
Control Subjects	50	52	18-55	25.3

Table 3.2. ACA ASSAY

Details of patients and control subjects

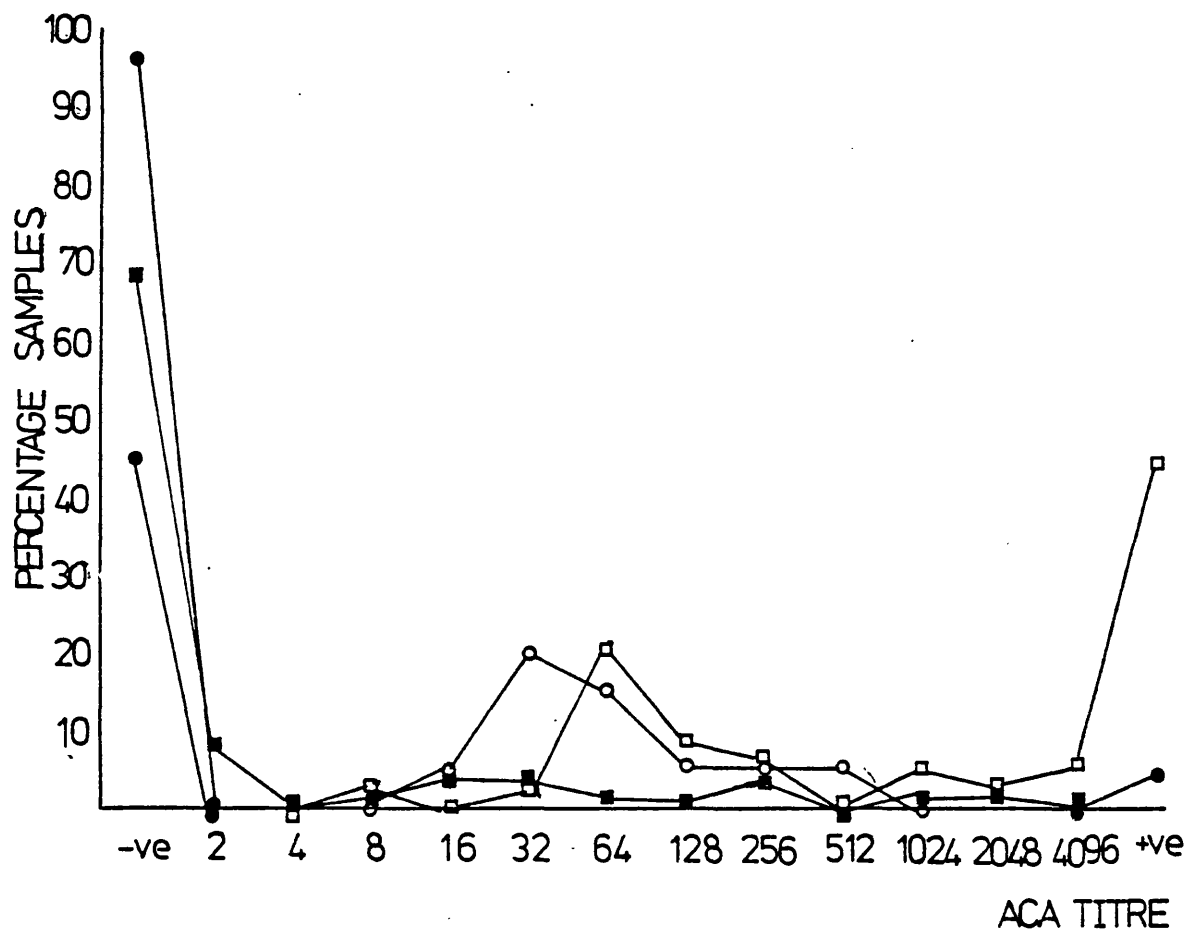


FIGURE 3-2

PERCENTAGE DISTRIBUTION OF ACA TITRE IN CONTROL SUBJECTS, (●-●), AND PATIENTS WITH CROHN'S DISEASE, (■-■), RHEUMATOID ARTHRITIS, (○-○), AND ANKYLOSING SPONDYLITIS, (□-□).

Statistical comparison of ACA in SF from patients with RA and non-RA revealed no significant difference ($\chi^2 = 1.96$, $p > 0.2$). However, the group with non-RA was probably too small for tests of statistical significance.

3.3.3. C3 and C4 CONCENTRATIONS

C3 and C4 concentrations are expressed in mg% of serum or SF.

Details of patients, age range, mean age and number of samples tested with mean C3 and C4 concentrations ± 1 standard deviation (SD) are given in Table 3.3.

Comparative results of C3 determinations are given in Figure 3.3 and of C4 determinations in Figure 3.4., with mean ± 1 SD.

Both mean C3 (98.51 ± 31.51 mg%) and mean C4 (35.44 ± 12.74 mg%) levels in sera of patients with CD were raised when compared to mean C3 (74.94 ± 19.95 mg%) and mean C4 (27.61 ± 10.34 mg%) levels measured in sera from control subjects ($t = 5.27$, $p < 0.005$ and $t = 4.07$, $p < 0.005$, respectively).

Conversely, mean C3 (54.3 ± 8.81 mg%) and mean C4 (19.32 ± 4.63 mg%) levels in sera from patients with AS and mean C3 (51.8 ± 27.26 mg%) and mean C4 (10.74 ± 7.5 mg%) levels in SF from patients with RA were significantly lower than mean C3 and C4 levels in sera from control subjects ($t = 5.60$, $p < 0.005$; $t = 4.15$, $p < 0.005$ and $t = 4.26$, $p < 0.005$; $t = 7.81$, $p < 0.005$, respectively).

No significant differences were found in C3 or C4 levels in SF from patients with RA or non-RA ($t = 0.44$, $p > 0.25$ and $t = 0.53$, $p > 0.25$). The group of patients with non-RA was probably too small for tests of statistical significance.

Patient Group	No. of Subjects	No. of Samples	Age Range (Years)	Mean Age (Years)	Mean Conc. \pm 1 S.D.* (mg%)
C3	55	99	18-76	40	98.51 \pm 31.51
C4	55	95	18-76	40	35.44 \pm 12.74
C3	8	8	19-55	41	54.3 \pm 8.81
C4	5	5	19-47	37.2	19.32 \pm 4.63
C3	20	20	18-69	49	51.8 \pm 27.26
C4	17	17	18-69	50	10.74 \pm 7.5
C3	5	5	NOT KNOWN	NOT KNOWN	48.6 \pm 7.44
C4	6	6	NOT KNOWN	NOT KNOWN	12.46 \pm 7.36
C3	53	57	19-60	27.5	74.94 \pm 19.95
C4	51	53	19-60	27.5	27.61 \pm 10.34

* Standard Deviation

Table 3.3. C3 AND C4 CONCENTRATION

Details of patients and control subjects

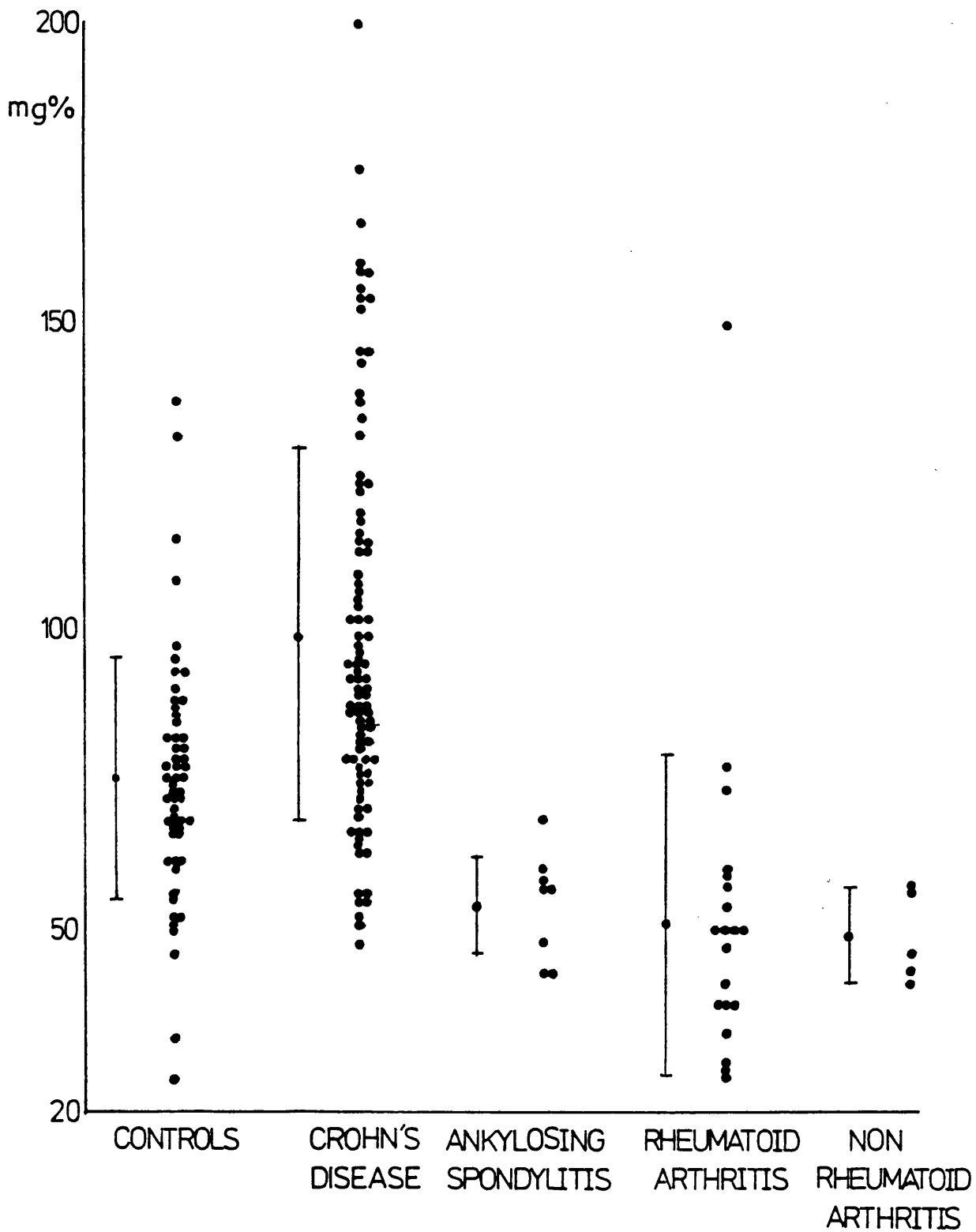


FIGURE 3-3

DISTRIBUTION OF COMPLEMENT C3 LEVELS WITH MEAN \pm 1
STANDARD DEVIATION

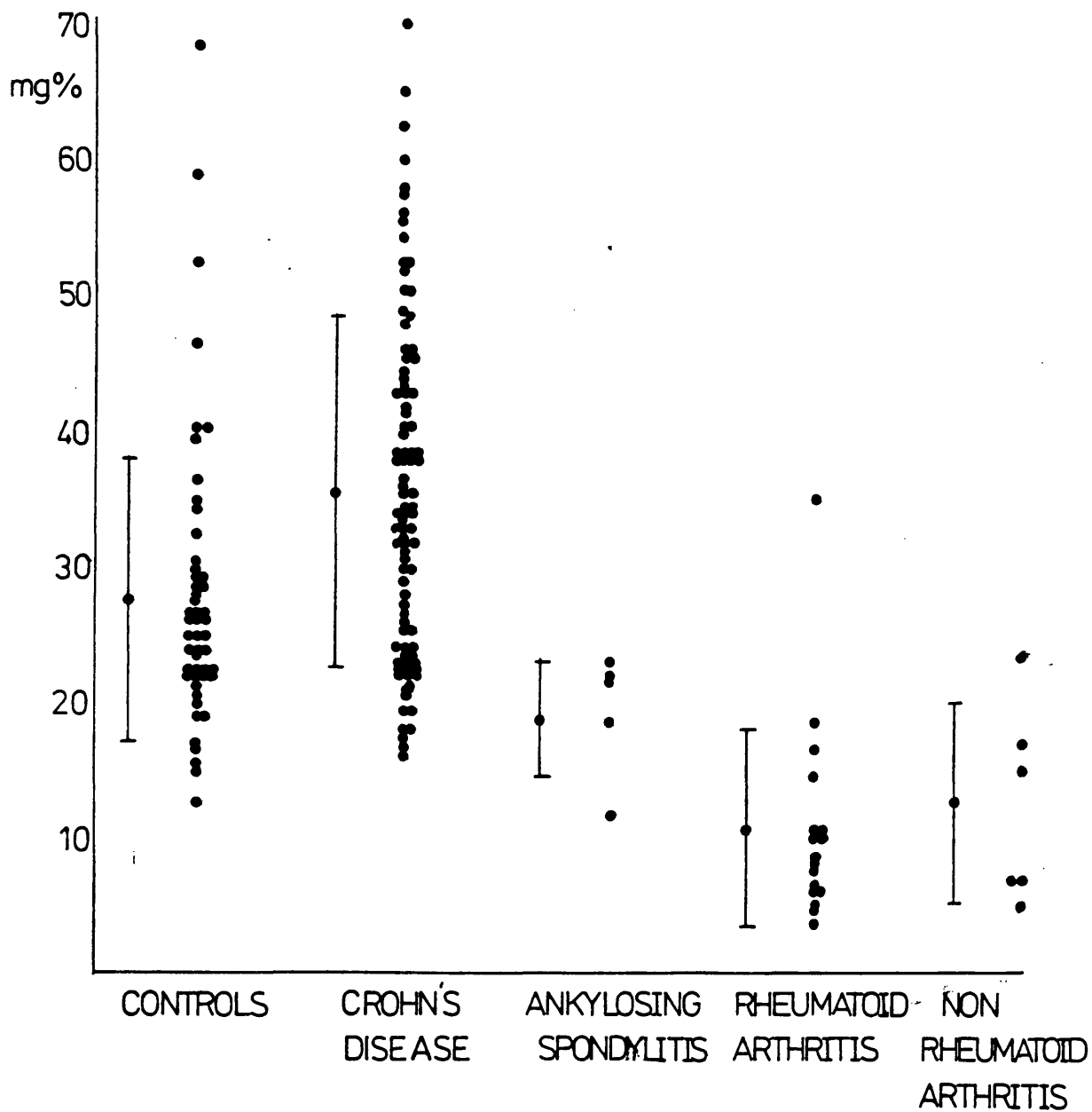


FIGURE 3-4

DISTRIBUTION OF COMPLEMENT C4 LEVELS , WITH MEAN \pm 1
STANDARD DEVIATION

There was a direct correlation between C3 and C4 levels in sera from patients with CD (Figure 3.5) ($r = 0.514$, $p < 0.001$). This was not seen in sera from control subjects ($r = 0.012$, $p > 0.10$).

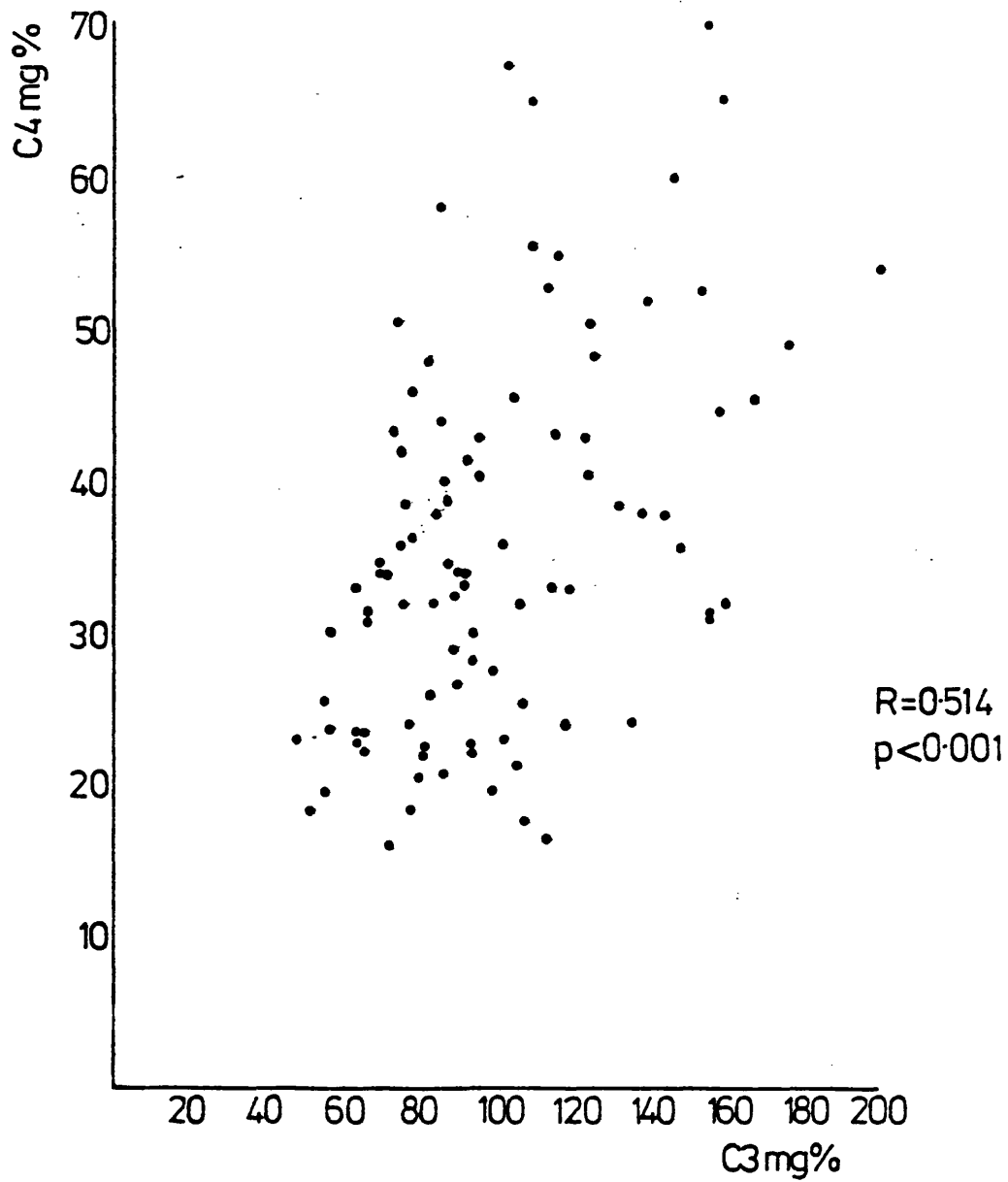


FIGURE 3.5

CORRELATION BETWEEN C3 AND C4 LEVELS IN 94 SERA
FROM PATIENTS WITH CROHN'S DISEASE

3.4. DISCUSSION

Evidence of circulating ICs detected by the ACA assay was found in 32.2% of sera from patients with CD. This is in close agreement with Hodgson, Potter and Jewell (1977a), who found such evidence in 27% of patients with CD using a similar ACA assay described by Johnson, Mowbray and Porter (1975). Johnson, Mowbray and Porter (1975) demonstrated the existence of circulating ICs in various pathological sera, not including CD, by ACA assay. They emphasised the importance of careful standardization of the assay, including standard storage methods, collection of samples, heat inactivation procedure and conditions of incubation with C'. This was confirmed by Neilsen and Svehag (1976).

In this study the experimental procedures and handling of samples were standardized as far as possible.

Evidence presented by Hodgson, Potter and Jewell (1977a) using aggregates of immunoglobulin G(IgG) indicated that the ACA test detects small ICs, 11 Svedbergs (s) in size, whilst Clq precipitation detects larger complexes, over 20s in size. Using the Clq precipitation technique, they found that only 3 out of 62 patients with CD showed evidence of large ICs. However, Doe, Booth and Brown (1973), using a different adaptation of the Clq precipitation technique found that 57% of patients with CD gave positive reactions. Clq is known to precipitate soluble ICs in agar gel (Agnello, Winchester and Kunkel, 1970), but only if aggregates are over 19s in size. Hay et al (1976) devised a solid phase radio-assay for Clq precipitation and suggested that larger aggregates would be detected by these Clq precipitation methods because they had a higher avidity for Clq than small aggregates. Although the evidence remains controversial, it appears that Clq

precipitation techniques detect ICs of a larger size whereas the ACA method may detect smaller ICs. With normal hepatic function in animal experiments (Mannik, Haakenstad and Arend, 1974) large ICs are rapidly cleared from the circulation by the reticulo-endothelial system (RES) whilst smaller ICs continue to circulate. This has been suggested to occur in humans (Hodgson, Potter and Jewell, 1977a) without liver disease and therefore a method of detection of smaller ICs may be of more use than one that detects larger ICs. Since ACA depends on activation of the whole C' sequence it should theoretically detect ICs which activate the C' system by both the alternative and classical pathways (Neilsen and Svehag, 1976) whereas Clq precipitation methods would only detect ICs which activate the C' sequence by the classical pathway via fixation of Clq. That is, only ICs containing IgG or IgM would be detected by Clq precipitation. As the inflamed mucosa in patients with CD contains increased numbers of Ig-producing cells (Baklien and Brandtzaeg, 1975) it seems likely that ICs may be formed in the mucosa itself (Hodgson, Potter and Jewell, 1977a) and may contain IgA.

Using the ACA method described, no evidence of ICs was found in sera from control subjects, apart from two sera which were strongly ACA positive. This activity may be due to misleading causes because both these samples were obtained on the same day in an extremely fatty state and on subsequent testing of second samples from these two individuals they proved to have negative ACA. It is possible that excessive fat content may have interfered with the ACA test. No other sera had such a fatty appearance. Apart from this discrepancy, all serum samples were collected and handled under identical conditions, with the exception of sera from patients with AS which had been stored for slightly longer periods, up to 12 months compared to

a maximum of 2 months for sera from control subjects and patients with CD and SF from patients with RA. Sera from patients with AS proved to have considerably greater ACA than other sera but not SF from patients with RA. All serum samples from patients with AS had some ACA, 44% being strongly positive indicating a high content of ICs. 55% of SF from patients with RA showed evidence of containing ICs. This is in agreement with previous experiments (Zvaiffler, 1974; Barnett and MacLennan, 1972; Zubler et al, 1976) using various methods of IC detection. Of the five non-RA SF tested, only one showed any ACA. Despite the small size of this group, a lesser percentage showed evidence of ICs in the SF than did the group of patients with RA.

It could be inferred that sera from patients with AS showed greater evidence of ICs due to false positive results caused by aggregation of IgG during the longer period of storage. However, all samples were incubated for 60 minutes at 56°C prior to testing for ACA, and this treatment has been shown to reduce background ACA caused by aggregated IgG (Johnson, Mowbray and Porter, 1975; Neilsen and Svehag, 1976).

Other factors affecting ACA must be considered. Inhibitors of activated C' components e.g. C1 inhibitor (C1-INH) and conglutinin-activation factor (KAF) may contribute to giving false positive ACA (Hodgson, Potter and Jewell, 1977a). Such inhibitors are destroyed by heating to 56°C for 60 minutes, so should not affect ACA under the conditions of these experiments.

When data from the CH₅₀ determinations were examined there was little apparent difference between patients with CD and control subjects. Statistical analysis using the Kolmogorov-Smirnov test confirmed no significant difference between the two groups.

Reduced CH_{50} titre has been demonstrated in SF from patients with RA (Pekin and Zvaiffler, 1964; Ruddy and Austen, 1970) and the results presented here are in agreement with that. Half of the samples tested had no detectable CH_{50} titre. This indicates that C' may be consumed by ICs. Similarly, 55% of sera from patients with AS showed no CH_{50} titre indicating a high content of ICs. Statistical comparison with CH_{50} titres in sera from control subjects showed CH_{50} titre to be significantly lower in sera from patients with AS and in SF from patients with RA. When distribution of CH_{50} titre in SF from patients with RA was compared to that in SF from 6 patients with non-RA the difference was not significant.

Estimation of CH_{50} titre is an indirect test for the presence of ICs as a low titre may not necessarily be due to ICs and can be affected by many factors, such as diminished activity of one or more components of the C' system or an increased amount of inhibitor or inactivator. However, taken together with ACA titres of the same patient group conclusions can be drawn. As can be seen from the results, patient groups with high ACA also tend to have lower CH_{50} distributions. When individual C' components C3 and C4 were measured a more striking difference between the groups emerged. Sera from patients with AS had low CH_{50} titres and also significantly reduced mean C3 and C4 concentrations when compared to control values. C3 and C4 levels in SF from patients with RA were markedly reduced. Direct comparisons cannot be made between sera from control subjects and SF. Ruddy and Austen (1970) showed that C3 and C4 concentrations in SF were significantly lower in subjects with seropositive RA than in subjects with other degenerative joint diseases or seronegative RA. No significant difference was found here between C3 or C4 levels in SF from patients with RA or non-RA.

This could be due to the fact that there were only 6 patients with non-RA and the patients with RA were not divided into seropositive and seronegative sub-groups.

In contrast, sera from patients with CD had comparatively normal CH_{50} titres and raised ACA titres but the mean C3 and C4 concentrations were significantly elevated when compared to control values. Some workers have also found raised C3 and C4 levels in CD (Hodgson, Potter and Jewell, 1977b) whereas others have reported them to be near normal (Teisberg and Baklien, 1974; Teisberg and Gjone, 1975). It has been suggested that rises in C3 and C4 levels could be a result of non-specific inflammation (Hodgson, Potter and Jewell, 1977b) but it has been argued that since raised levels are also found in patients in remission (Ward and Eastwood, 1974) C' activation must be involved in the immunopathology of the disease. There was no significant difference between C3 and C4 levels in sera with positive or negative ACA titre ($t = 0.854$, $p > 0.25$ and $t = 0.534$, $p > 0.25$, respectively). C3 and C4 levels were significantly elevated in both cases, suggesting this is not just an effect of inflammation but an effect of an ongoing immunological process. Similar observations have been made with elevated C3 proactivator (C3 proA) levels in patients with CD in remission (Feinstein et al, 1976) suggesting involvement of the alternate pathway of C' activation. In the results presented here, since both C3 and C4 levels were raised the classical pathway of C' activation is implicated.

3.5 CONCLUSION

Many methods of IC detection are available, none of which is able to detect all ICs. Ig content, size and degree of lattice formation in the complex are all important. The method used, measurement of ACA, implicated ICs in all of the sera tested from patients with AS, 55% of SF from patients with RA and also in 32.2% of sera from patients with CD. Control sera were negative in comparison. This method is considered to be a reliable and sensitive assay, providing conditions of experimentation and handling of samples are carefully standardized (Johnson, Mowbray and Porter, 1975; Neilsen and Svehag, 1976). It is possible that other methods may detect complexes of different sizes in pathological sera. CH_{50} titre was normal in sera from patients with CD but individual C' components C3 and C4 were found to be raised. This could be due to reduced C3 metabolism or increased C' synthesis possibly as a feedback mechanism to compensate for increased C' activation by ICs. In order to investigate this possibility, C3 metabolism was examined by estimation of C3 inactivation products (C3i). This work is described in Chapter V.

CHAPTER 4

COMPLEMENT AND IMMUNE COMPLEX STUDIES

IN RELATION TO PARAMETERS OF DISEASE

IN PATIENTS WITH CROHN'S DISEASE.

4.1 INTRODUCTION

The work reported in Chapter III showed that 32.2% of sera from patients with Crohn's disease (CD) had evidence of circulating immune complexes (IC) as shown by assessment of anticomplementary activity (ACA). ACA was not found in an age and sex-matched population of control subjects. There was no difference in CH_{50} levels between the two groups, but levels of complement (C') components C3 and C4 were both raised significantly in patients with CD.

Data obtained from work described in Chapter III was analysed in order to see if the variations observed could be ascribed to factors of the disease, namely disease activity, duration of disease and drug therapy. Attempts have been made previously by other workers to correlate such parameters with the presence of ICs in pathological sera. Early work concentrated on variations in C' levels in sera from patients with ulcerative colitis (UC) (Thayer and Spiro, 1963; Fletcher, 1965) but later sera from patients with CD were examined for correlations between circulating ICs and the parameters mentioned above, (Jewell and MacLennan, 1973; Teisberg and Gjone, 1975; Hodgson, Potter and Jewell, 1977a and 1977b).

Analysis of CH_{50} and ACA assay and C3 and C4 estimations in sera from patients with CD as described in Chapter III was carried out according to:

1. Duration of disease;
2. Steroid therapy;
3. Disease activity.

4.1.1 DURATION OF DISEASE

Variation in C' and ICs was examined according to the duration of disease process in the following categories:-

- (i) less than 2 years;
- (ii) 2-5 years;
- (iii) 5-10 years;
- (iv) over 10 years.

4.1.2 STEROID THERAPY

Corticosteroid therapy is a major therapeutic modality in the management of inflammatory and immunologically mediated diseases. In the cases of CD studied the most commonly used corticosteroid was prednisolone. The dose varied from 10 mg. three times daily (t.d.s.) to as little as 1 mg. per day. Generally patients were started on a high dose, to control an acute exacerbation, which was gradually reduced over a few months as the symptoms subsided.

Azathioprine (AZA) is often used in conjunction with Pred therapy in CD. Again dosage is altered according to severity of symptoms and in the patients of this study varied from 50 mg. t.d.s. to 25 mg. daily.

Incidence of ICs and raised C' in sera of patients with CD was examined according to the following categories of steroid therapy.

- (i) no steroids;
- (ii) no steroids for at least 2 years;
- (iii) steroids.

A variety of other drugs are also used in the management of CD. including codeine phosphate, salazopyrine and isogel. Their possible effects on the immune system will be discussed.

4.1.3 DISEASE ACTIVITY

Disease activity was assessed by clinical, radiological and histological findings based on published criteria (Truelove and Witts, 1955; de Dombal et al, 1974).

Results of IC detection and raised C' in the sera of patients with CD were analysed according to whether the patients had:

- (i) active disease;
- (ii) quiescent disease;

4.2 PATIENTS AND METHODS

4.2.1 PATIENTS

Details of patients with CD and number of serum samples analysed for each parameter measured are given in Table 4.1.

4.2.2 METHODS

The methods of estimation of CH_{50} and ACA titre and C3 and C4 concentrations are described in Chapter II.

All blood samples were collected into plain bottles and allowed to clot at room temperature. The serum was spun off within two hours of collection and dispensed into 0.5 ml. aliquots which were stored immediately at -70°C . Freezing and thawing of samples was avoided, a fresh aliquot being used for each test.

4.2.3 STATISTICS

The Kolmogorov-Smirnov two sample χ^2 test (Seigel) and students t-test were used to determine whether the CH_{50} and ACA results and the C3 and C4 estimations, respectively, differed in the various groups of patients with CD.

Parameter Measured	No of Subjects	No. of Sera Tested for				Age Range (Years)	Mean Age (Years)
		THC	ACA	C3	C4		
D U R A T I O N S E F <2 years 2-5 years 5-10 years >10 years	21	34	35	28	27	18-76	43
	9	20	18	16	15	19-43	31.3
	21	30	26	29	29	21-73	41
	16	25	21	19	17	21-74	41.5
S T H E R R O I D Y none none for <2 yrs. steroids	24	31	27	26	26	21-74	43.4
	7	10	9	7	6	27-49	35.3
	31	68	65	60	57	18-76	40.7
A C T I V E Quiescent Active	52	79	69	66	63	19-76	41.6
	24	28	31	25	24	18-76	41.1

Table 4.1. Details of patients with Crohn's Disease and the parameters measured

4.3 RESULTS

The results of various assays on sera from patients with CD with different duration disease, steroid therapy and disease activity are presented as follows:-

4.3.1 DURATION OF DISEASE

The effect of duration of disease on C' and IC levels were studied in 82 patients with CD. The patients were divided into four categories of disease duration as described in the introduction.

4.3.1.1. Total Haemolytic Complement (THC)

THC has been defined in Chapter II.

Figure 4.1. illustrates the comparative percentage distribution of CH_{50} titres within the four categories of duration of disease process.

Statistical analysis of the results revealed no difference in distribution of CH_{50} titre between any of the groups of disease duration, (Table 4.2).

4.3.1.2. Anticomplementary Activity (ACA)

ACA has been defined in Chapter II.

Figure 4.2 illustrates the comparative percentage distribution of ACA titre within the four categories of duration of disease process.

Statistical analysis of the results revealed no relationship between distribution of ACA titre and disease duration (Table 4.2).

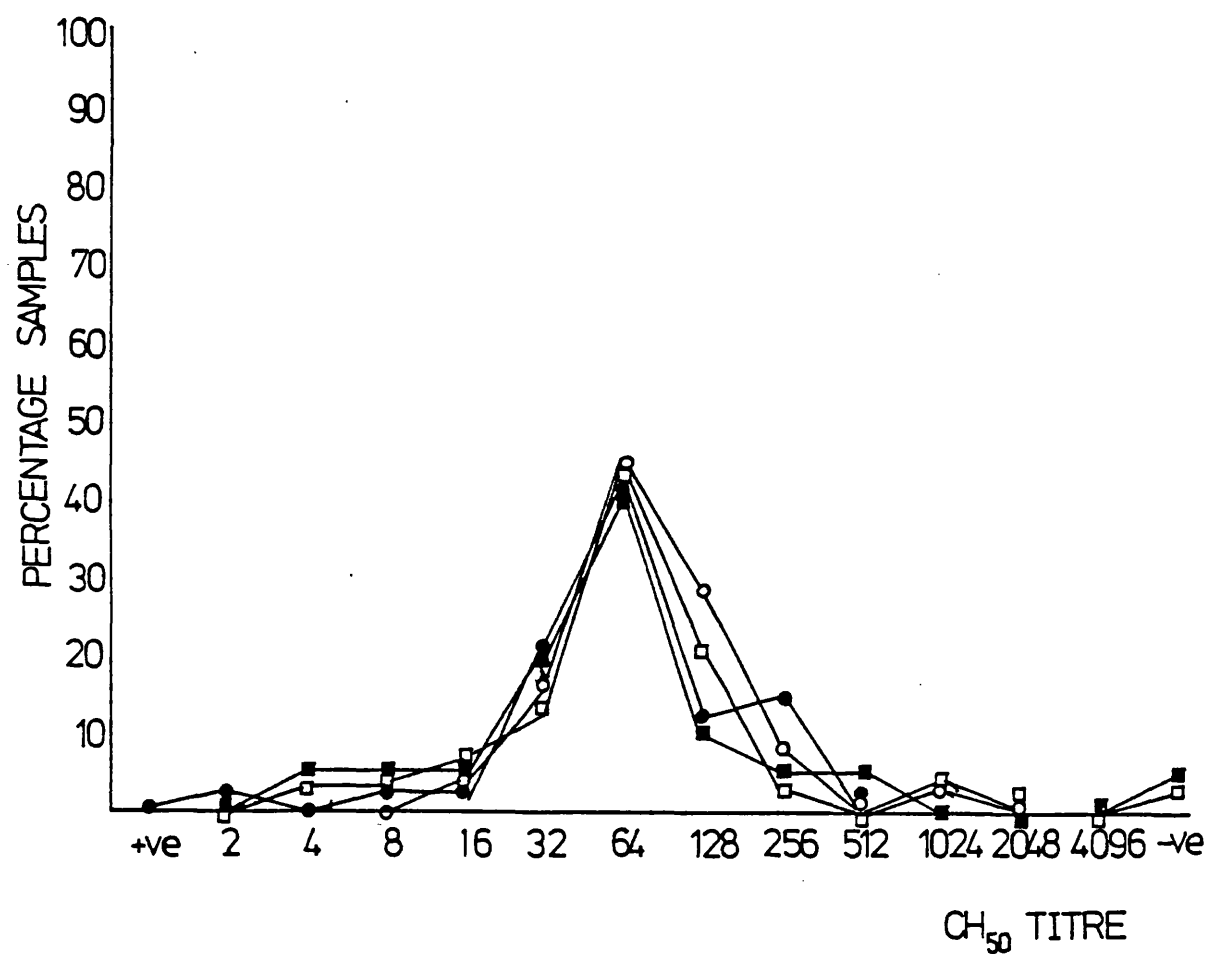


FIGURE 4.1

PERCENTAGE DISTRIBUTION OF CH₅₀ TITRE IN SERA FROM CROHN'S DISEASE PATIENTS WITH DURATION OF DISEASE

- (i) LESS THAN 2 YEARS (●-●)
- (ii) 2-5 YEARS (■-■)
- (iii) 5-10 YEARS (□-□)
- (iv) MORE THAN 10 YEARS (○-○)

Duration of Disease	CH ₅₀		ACA	
	x ²	p	x ²	p
<2 years-2-5 years	0.192	>0.1	0.416	>0.5
<2 years-5-10 years	0.371	>0.5	1.194	>0.1
<2 years->10 years	0.51	>0.1	0.577	>0.1
2-5 years-5-10 years	0.335	>0.5	1.122	>0.1
2-5 years->10 years	1.00	>0.1	0.88	>0.1
5-10 years->10 years	0.47	>0.1	2.59	>0.1
Steroid Therapy	CH ₅₀		ACA	
	x ²	p	x ²	p
No steroids - no steroids<2 years	1.788	>0.1	0.30	>0.5
No steroids<2 years - steroids	1.29	>0.1	0.715	>0.1
Steroids - no steroids	1.52	>0.1	0.182	>0.5
Disease Activity	CH ₅₀		ACA	
	x ²	p	x ²	p
Active - quiescent disease	1.12	>0.1	1.08	>0.1

Table 4.2. Summary of Kolmogorov-Smirnov X^2 test of significance

between CH₅₀ and ACA distribution in sera of

patients with CD and duration of disease, steroid

therapy and disease activity

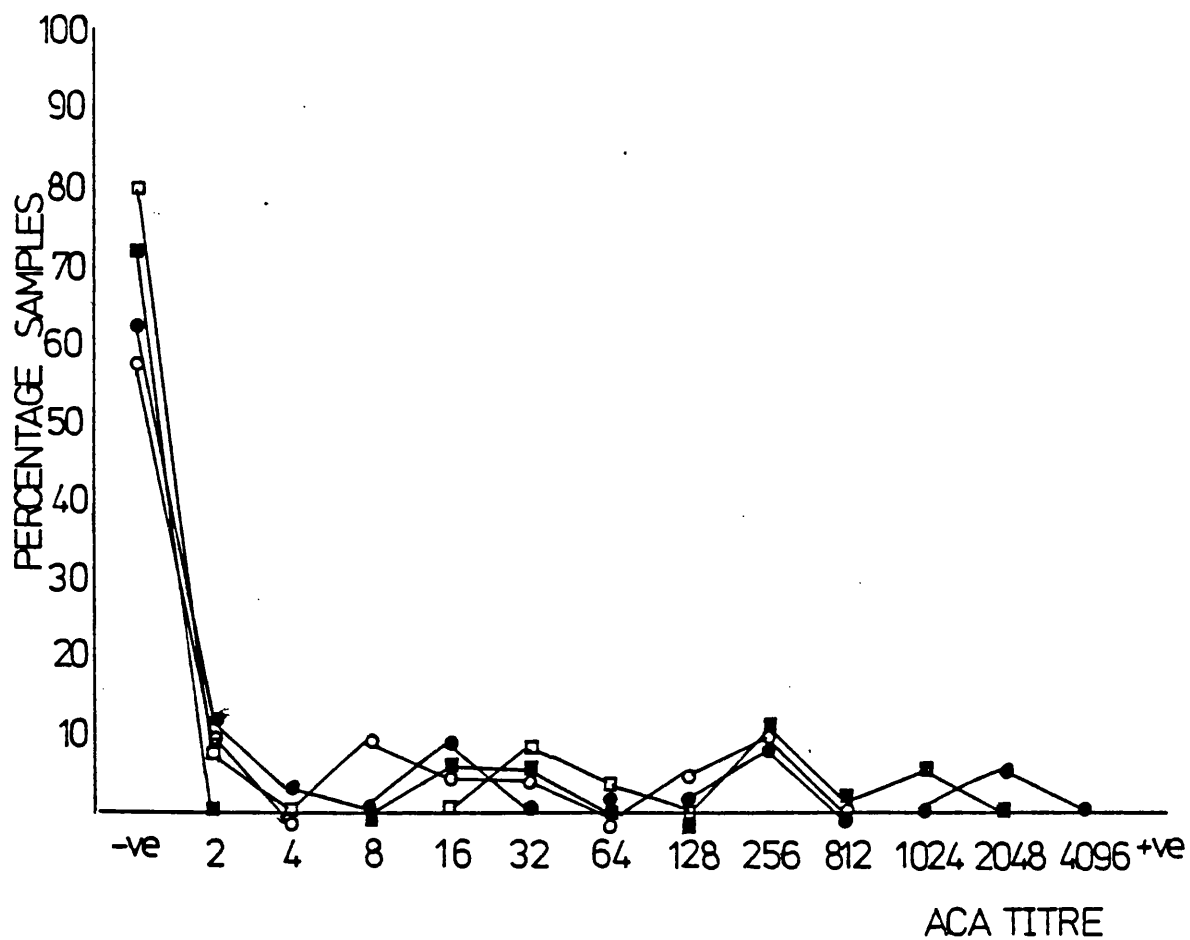


FIGURE 4.2

PERCENTAGE DISTRIBUTION OF ACA TITRE IN SERA FROM CROHN'S DISEASE PATIENTS WITH DURATION OF DISEASE

- (i) LESS THAN 2 YEARS (●-●)
- (ii) 2-5 YEARS (■-■)
- (iii) 5-10 YEARS (□-□)
- (iv) MORE THAN 10 YEARS (○-○)

4.3.1.3. C3 and C4 Concentration

Complement components C3 and C4 are expressed in mg% of serum.

Figures 4.3 and 4.4 illustrate the comparative distributions with mean concentrations ± 1 standard deviation (SD) of C3 and C4 determinations, respectively.

Statistical analysis of mean C3 and C4 concentrations between the four groups of disease duration is given in Table 4.3.

As can be seen from Table 4.3., C3 and C4 concentrations were significantly raised in the group with less than (<) 2 years disease duration when compared to concentrations in the group with 2-5 years disease duration. C3 and C4 concentrations were also significantly raised in the group with 5-10 years disease duration when compared to concentrations in the group with 2-5 years disease duration. C3 concentrations in the group with 5-10 years disease duration were also significantly raised when compared to concentrations in the group with over (>) 10 years disease duration. No other significant differences were observed.

The highest mean C3 and C4 concentrations were found in the group with disease duration of 5-10 years and the lowest in the group with disease duration of 2-5 years.

Mean C3 and C4 concentrations in all groups were raised when compared to the control values quoted in Chapter III, this being significant in all cases with the exception of the mean C4 concentration in the group with 2-5 years disease duration which was raised when compared to the control mean, but not significantly so.

No direct relationship existed between duration of disease and C3 or C4 concentrations.

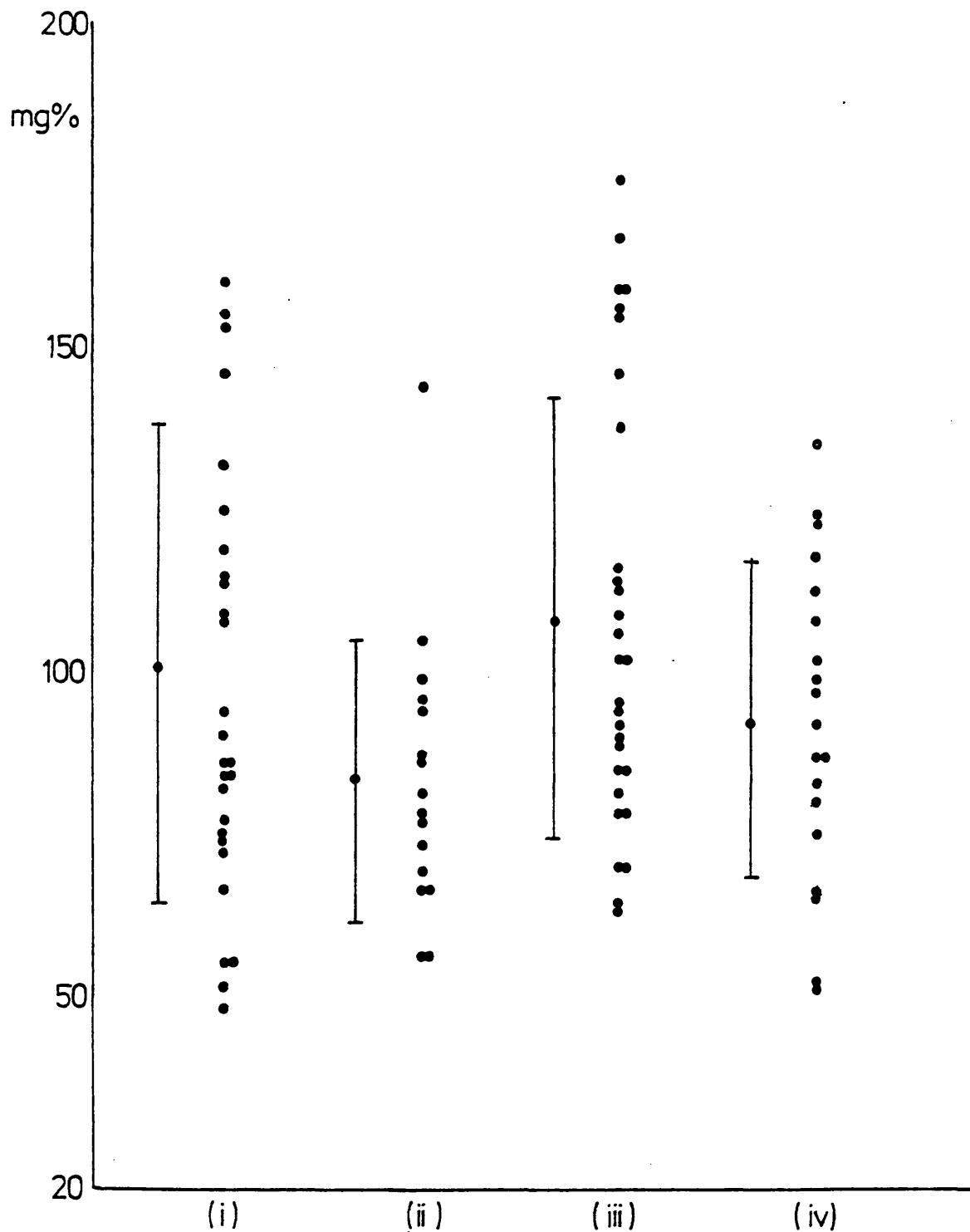


FIGURE 4-3

DISTRIBUTION OF SERUM COMPLEMENT C3 IN CROHN'S DISEASE
 PATIENTS WITH DURATION OF DISEASE : (i) LESS THAN 2 YEARS

(ii) 2-5 YEARS

(iii) 5-10 YEARS

WITH MEAN \pm 1 STANDARD DEVIATION.

(iv) MORE THAN 10 YEARS

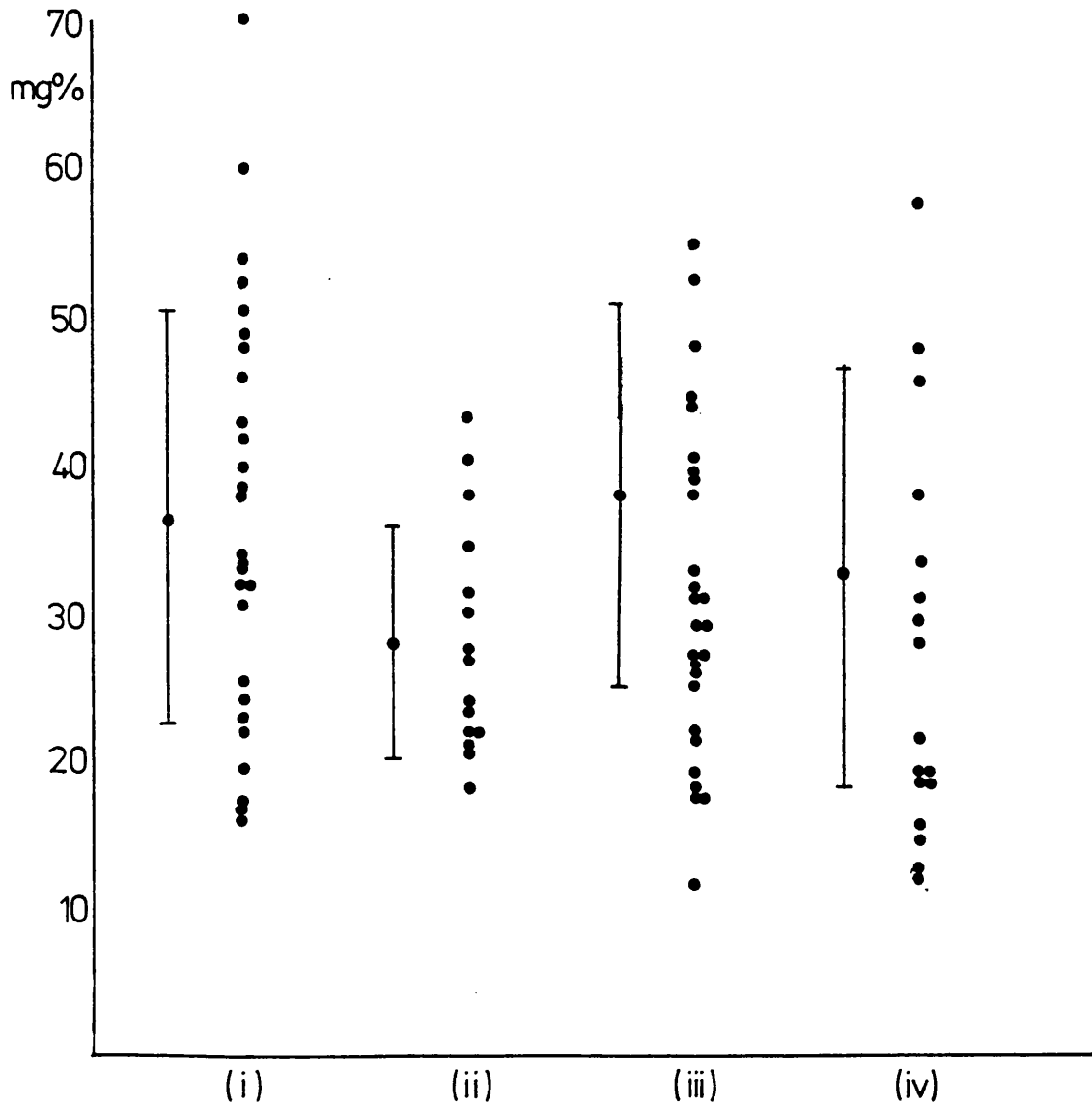


FIGURE 4.4

DISTRIBUTION OF SERUM COMPLEMENT C4 IN CROHN'S DISEASE
 PATIENTS WITH DURATION OF DISEASE (i) LESS THAN 2 YEARS
 (ii) 2-5 YEARS
 (iii) 5-10 YEARS
 (iv) MORE THAN 10 YEARS

WITH MEAN \pm 1 STANDARD DEVIATION

Duration of Disease	C3 mg% \pm 1S.D.		C4 mg% \pm 1S.D.	
<2 years	100.54	\pm 37.72	36.69	\pm 14.07
2-5 years	83.31	\pm 21.87	28.32	\pm 7.81
5-10 years	108.69	\pm 33.82	38.12	\pm 12.91
>10 years	92.47	\pm 24.30	32.42	\pm 14.31
Groups Compared	t	p	t	p
<2 years-2-5 years	1.91	<0.05	2.48	<0.01
<2 years-5-10 years	0.84	>0.1	0.39	>0.25
<2 years->10 years	0.87	>0.1	1.00	>0.1
2-5 years-5-10 years	2.99	<0.005	3.13	<0.005
2-5 years->10 years	1.12	>0.1	1.42	>0.1
5-10 years->10 years	1.89	<0.05	1.42	>0.1

Table 4.3. Mean C3 and C4 concentrations in sera from patients with Crohn's disease and different duration of disease process with significance of difference of means between groups

4.3.2 STEROID THERAPY

The effect of steroid therapy on C' and IC levels was studied in 62 patients with CD. The patients were divided into three categories of steroid therapy as described in the introduction.

4.3.2.1. Total Haemolytic Complement

THC has been defined in Chapter II.

Figure 4.5. illustrates the comparative percentage distribution of CH_{50} titres within the three categories of steroid therapy.

Statistical analysis of the results revealed no difference in distribution of CH_{50} titre between any of the groups of steroid therapy (Table 4.2.).

4.3.2.2. Anti-complementary Activity

ACA has been defined in Chapter II.

Figure 4.6 illustrates the comparative percentage distribution of ACA titres within the three categories of steroid therapy.

Statistical analysis of the results revealed no relationship between distribution of ACA titre and steroid therapy. (Table 4.2.).

4.3.2.3. C3 and C4 Concentration

Complement components C3 and C4 are expressed in mg% of serum.

Figures 4.7. and 4.8. illustrate the comparative distributions with mean ± 1 SD of C3 and C4 determinations respectively.

Statistical analysis of mean C3 and C4 concentrations between the three groups of steroid therapy is given in Table 4.4.

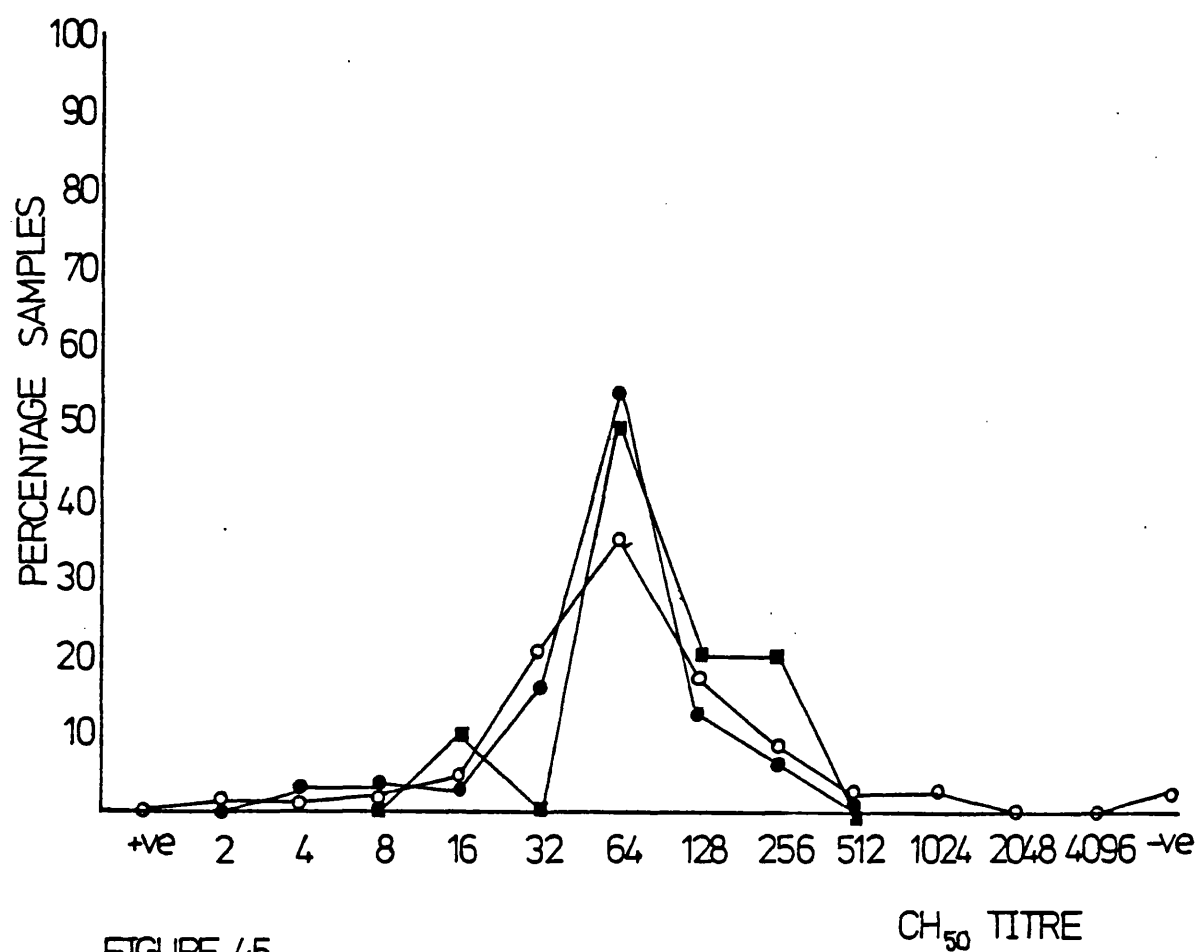


FIGURE 4.5

PERCENTAGE DISTRIBUTION OF CH₅₀ TITRE IN SERA FROM CROHN'S DISEASE PATIENTS TREATED WITH : NO STEROIDS (●-●); NO STEROIDS FOR AT LEAST 2 YEARS, (□-□); STEROIDS (○-○).

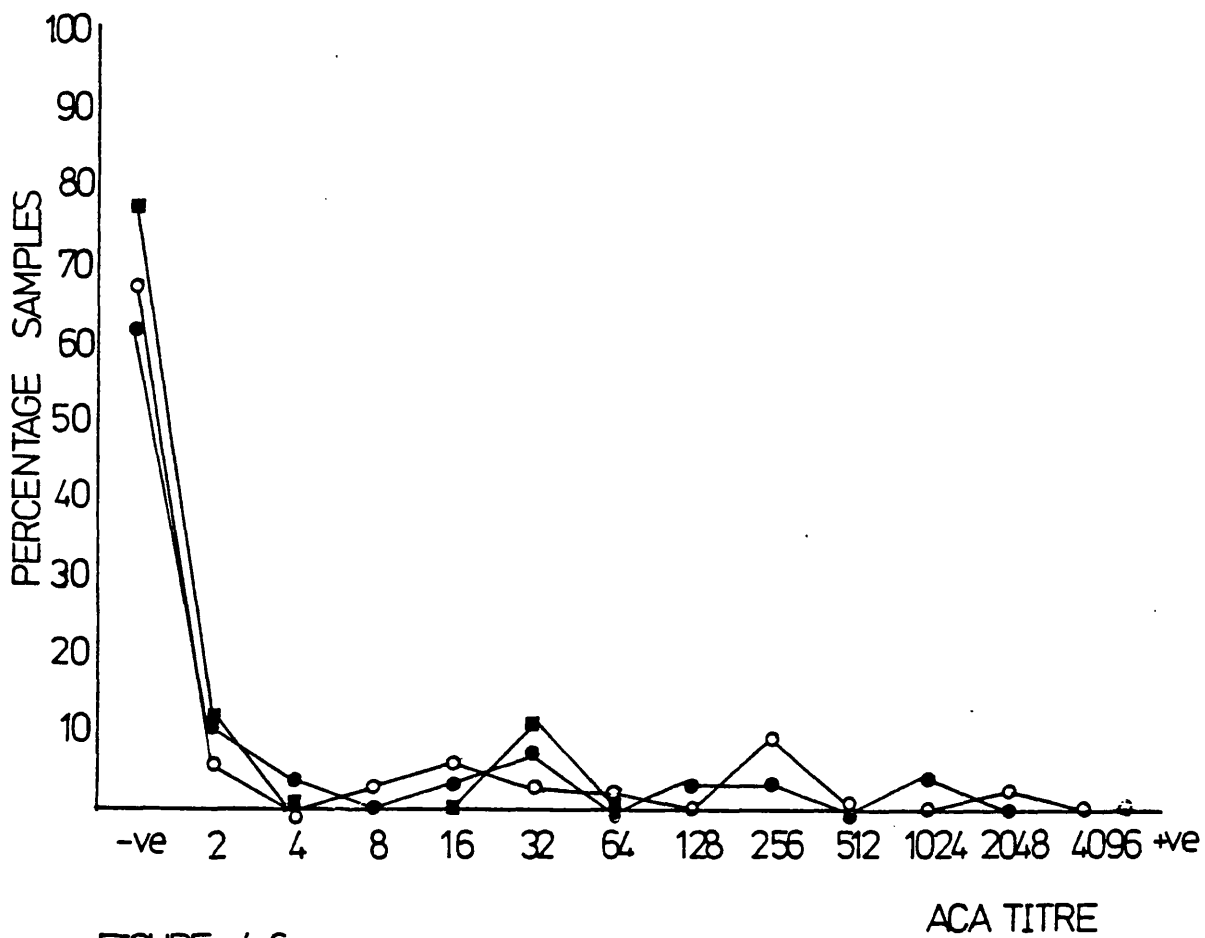


FIGURE 4.6

PERCENTAGE DISTRIBUTION OF ACA TITRE IN SERA FROM CROHN'S DISEASE PATIENTS TREATED WITH : NO STEROIDS, (●-●); NO STEROIDS FOR AT LEAST 2 YEARS, (■-■); STEROIDS, (○-○).

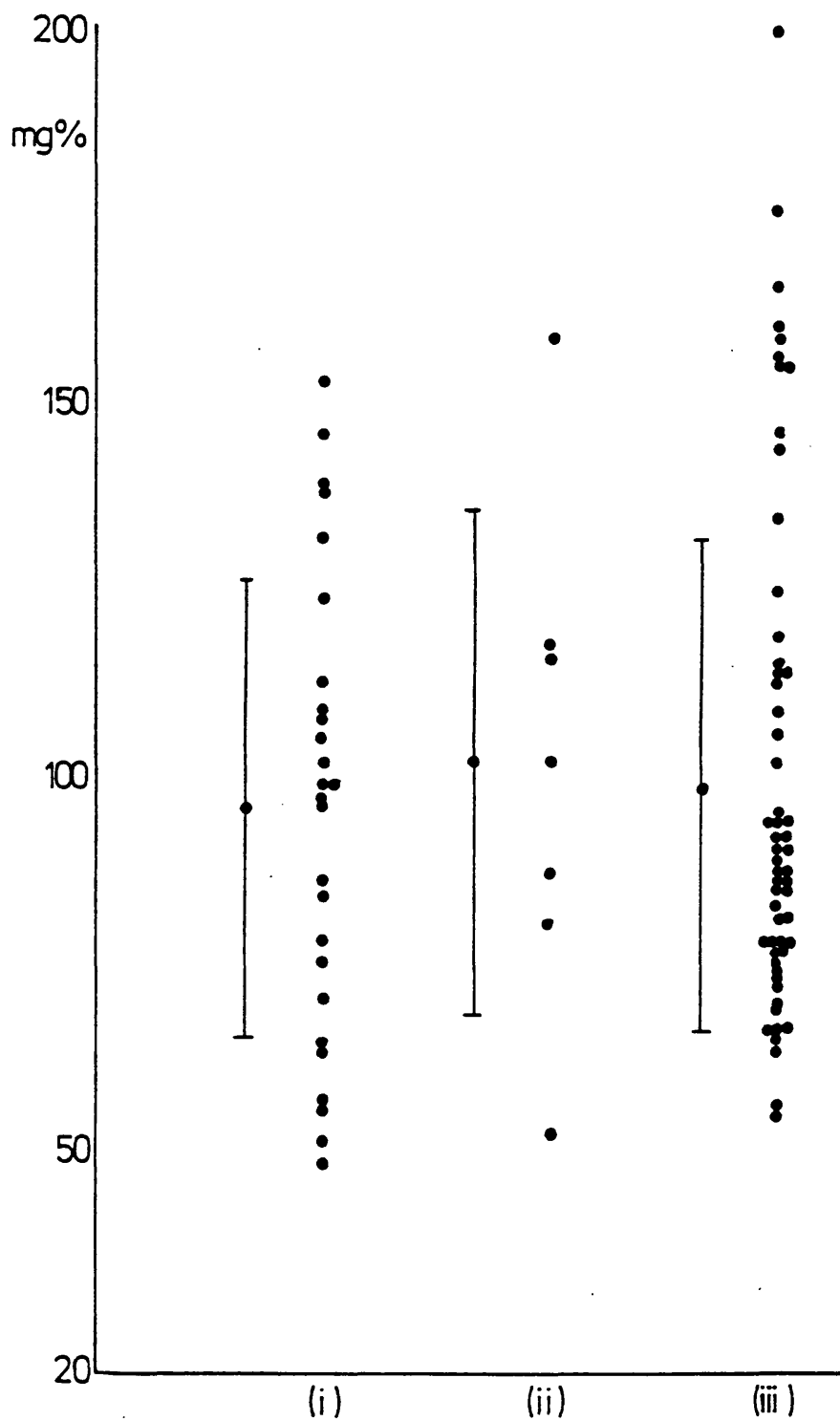


FIGURE 4.7

DISTRIBUTION OF SERUM COMPLEMENT C3 IN CROHN'S DISEASE
PATIENTS TREATED WITH: (i) NO STEROIDS

(ii) NO STEROIDS FOR AT LEAST 2 YEARS

(iii) STEROIDS

WITH MEAN ± 1 STANDARD DEVIATION

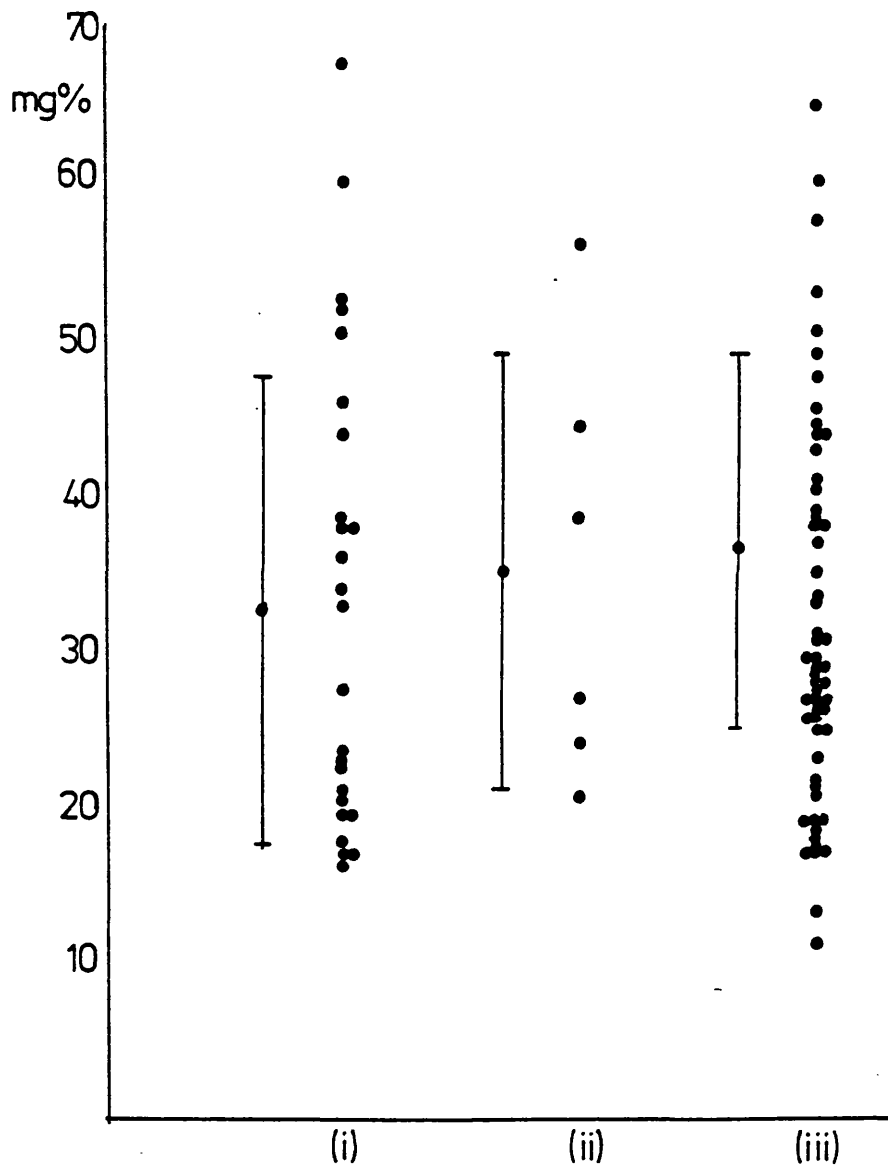


FIGURE 4.8

DISTRIBUTION OF SERUM COMPLEMENT C4 IN CROHN'S
DISEASE PATIENTS TREATED WITH (i) NO STEROIDS
(ii) NO STEROIDS FOR AT
LEAST 2 YEARS
(iii) STEROIDS

WITH MEAN \pm 1 STANDARD DEVIATION

Steroid Therapy	C3 mg% \pm 1 S.D.		C4 mg% \pm 1 S.D.	
No steroids	95.77 \pm 30.72		32.68 \pm 14.65	
No steroids<2 years	102.00 \pm 33.94		35.10 \pm 13.72	
Steroids	99.08 \pm 33.23		36.74 \pm 12.21	
Groups compared	t	p	t	p
No steroids - No steroids<2 years	0.53	>0.25	0.47	>0.25
No steroids<2 years - steroids	0.35	>0.25	0.49	>0.25
Steroids - No steroids	0.47	>0.25	1.36	>0.1

Table 4.4. Mean C3 and C4 concentrations in sera from patients with Crohn's disease and steroid therapy, with significance of difference of means between groups

No significant differences were found.

C3 and C4 concentrations were significantly raised in all groups when compared to control values quoted in Chapter III.

4.3.2.4. Other Drugs

A summary of other drugs used in the management of the patients with CD in this study is given in Table 4.5.

22 patients being treated with corticosteroids were also receiving AZA therapy, whereas, only 4 patients not on steroid therapy were being treated with AZA. Similar numbers of both patients who were on steroid therapy and those who were not, were also being treated with the other drugs listed.

A similar percentage of both patients with active and quiescent disease were receiving steroid therapy, i.e. 64%. More patients with active disease were also on AZA therapy; 23/24 compared to 45/56 of the patients with quiescent disease.

Drug	Drug Type	No. of Subjects on		
		No steroids	No steroids for 2 years	Steroids
Azathioprine	Immunosuppressant	2	2	22
Codeine Phosphate	Analgesic Smooth muscle relaxant	6	2	5
Salazopyrine	Sulphonamide	4	1	2
Isogel	Purgative	3	2	3
Others:				
Phenylbutazone	Analgesic, Anti-inflammatory			
Probanthine	Smooth muscle relaxant	3	0	1
Neocytamen	Vitamin B			
Metronidazole	Antiprotozoan			
No other drug		11	1	1

Table 4.5. Number of patients with Crohn's disease taking drugs other than steroids

4.3.3. DISEASE ACTIVITY

The effect of disease activity on C' and IC levels was studied in 76 patients with CD. The patients were divided into two categories of disease activity as described in the introduction.

4.3.3.1. Total Haemolytic Complement

THC has been defined in Chapter II.

Figure 4.9. illustrates the comparative percentage distribution of CH₅₀ titre within the two categories of disease activity.

Statistical analysis of the results revealed no difference in distribution of CH₅₀ titre between the two groups of disease activity (Table 4.2.).

4.3.3.2. Anti-complementary Activity

ACA has been defined in Chapter II.

Figure 4.10. illustrates the comparative percentage distribution of ACA titre within the two categories of disease activity.

Statistical analysis of the results revealed no relationship between distribution of ACA titre and disease activity (Table 4.2.).

4.3.3.3. C3 and C4 Concentrations

Complement components C3 and C4 are expressed in mg% of serum.

Figures 4.11. and 4.12. illustrate the comparative distributions with mean \pm 1 SD of C3 and C4 concentrations, respectively.

Statistical analysis of mean C3 and C4 concentrations between the two groups of disease activity is given in Table 4.6.

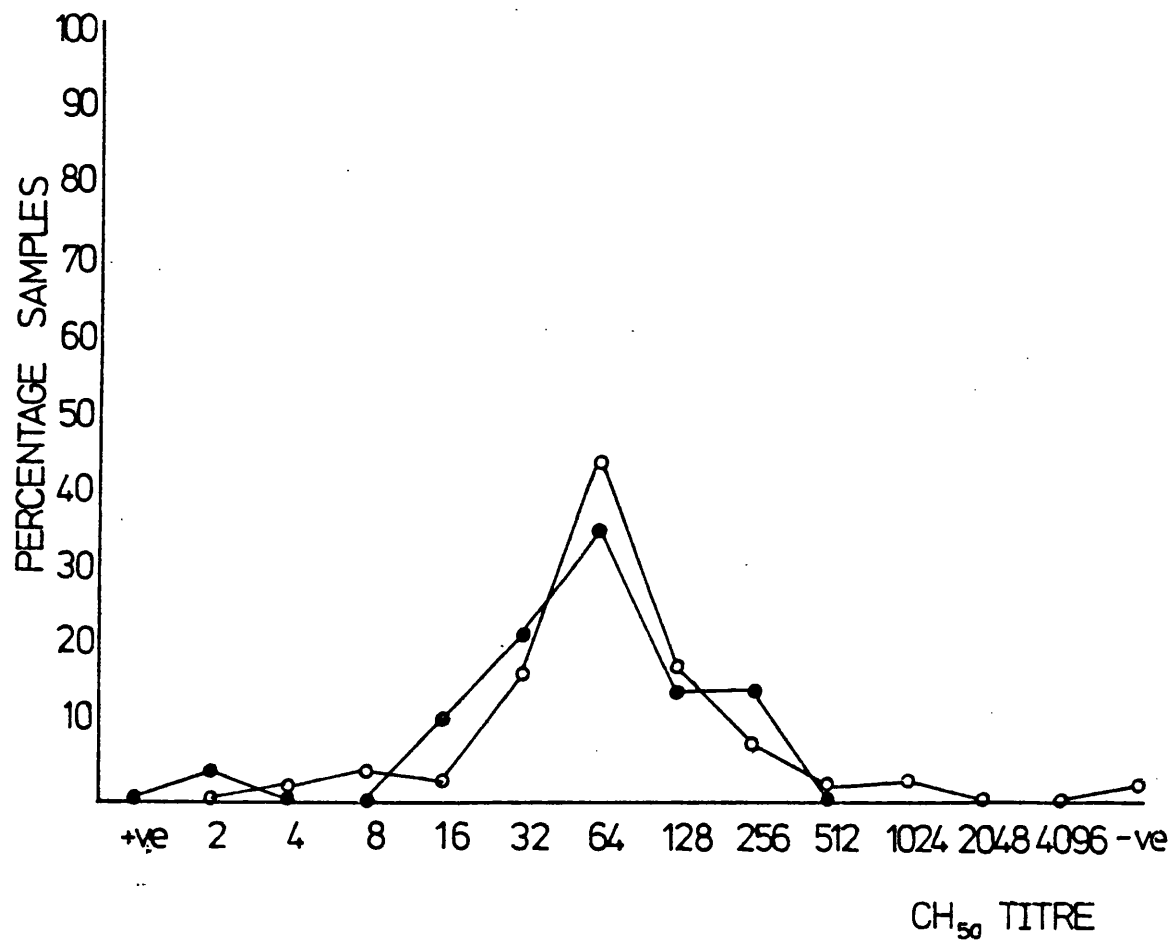


FIGURE 4.9

PERCENTAGE DISTRIBUTION OF CH₅₀ TITRE IN SERA OF CROHN'S DISEASE PATIENTS WITH ACTIVE DISEASE, (● - ●); OR QUIESCENT DISEASE, (○ - ○).

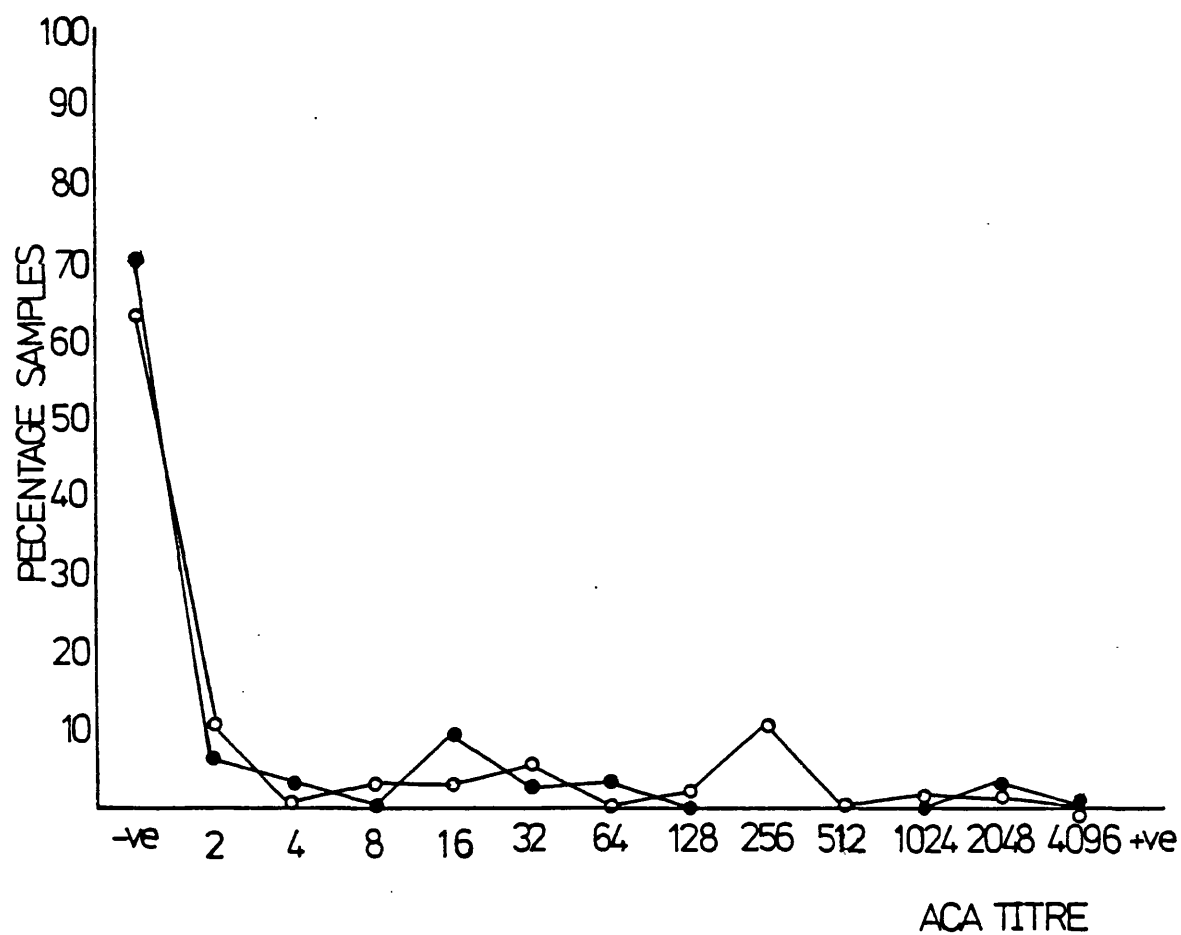


FIGURE 4.10

PERCENTAGE DISTRIBUTION OF ACA TITRE IN SERA OF CROHN'S DISEASE PATIENTS WITH : ACTIVE DISEASE, (●-●); OR QUIESCENT DISEASE, (○-○).

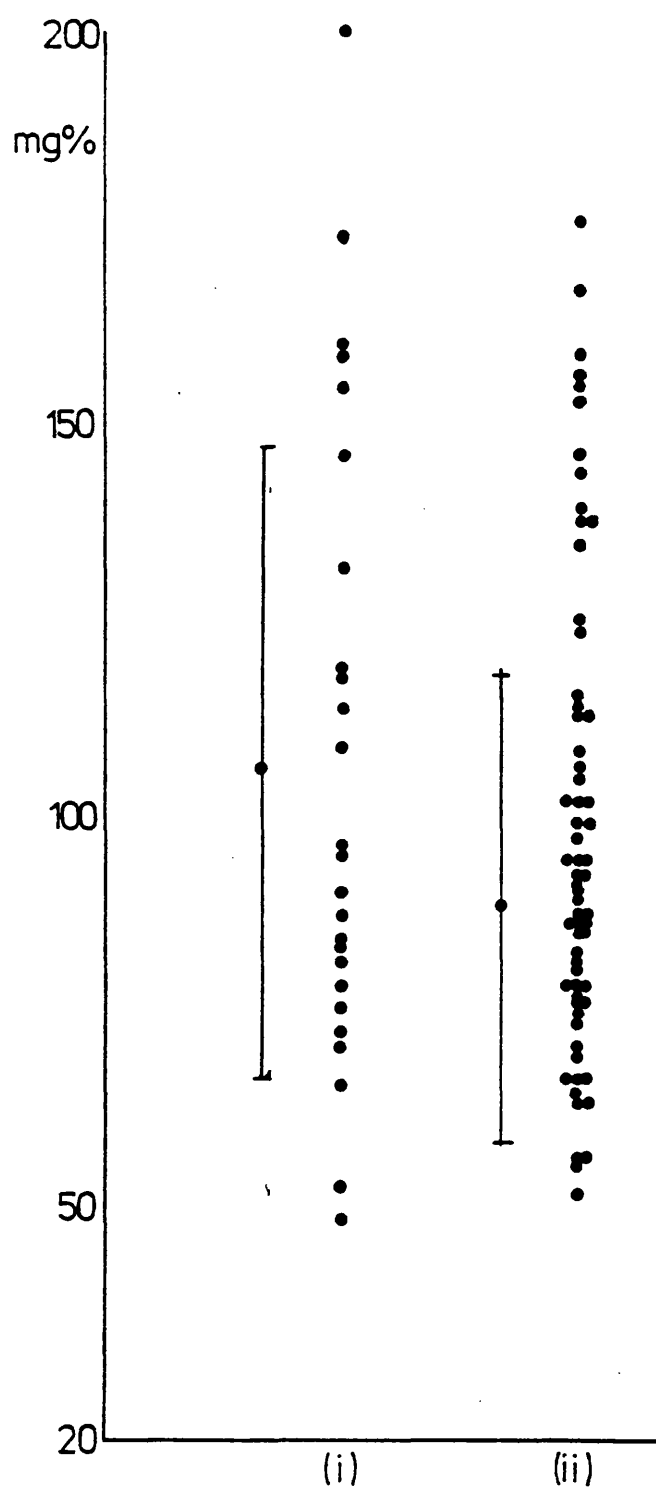


FIGURE 4-11

DISTRIBUTION OF SERUM COMPLEMENT C3 IN CROHN'S DISEASE
PATIENTS WITH : (i) ACTIVE DISEASE

(ii) QUIESCENT DISEASE

WITH MEAN \pm 1 STANDARD DEVIATION

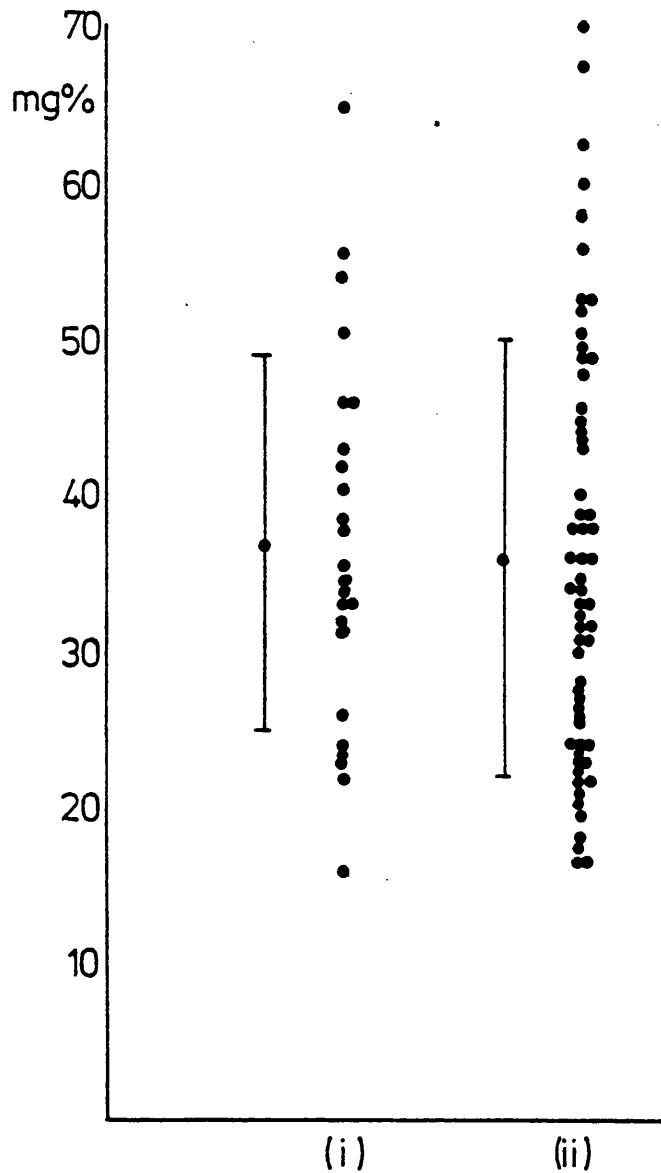


FIGURE 4.12

DISTRIBUTION OF SERUM COMPLEMENT C4 IN CROHN'S DISEASE
 PATIENTS WITH : (i) ACTIVE DISEASE
 (ii) QUIESCENT DISEASE
 WITH MEAN \pm 1 STANDARD DEVIATION

Disease Activity	C3 mg% \pm 1 S.D.		C4 mg% \pm 1 S.D.	
Active disease	106.48 \pm 40.24		37.00 \pm 11.92	
Quiescent disease	98.23 \pm 30.31		36.33 \pm 14.28	
Groups Compared	t	p	t	p
Active disease - Quiescent disease	1.10	>0.1	0.23	>0.25

Table 4.6. Mean C3 and C4 concentrations in sera from patients with Crohn's disease and disease activity, with significance of difference of means between groups

Mean C3 and C4 concentrations were higher in the group with active disease than in the group with quiescent disease, but this difference was not significant.

C3 and C4 concentrations were significantly raised in both groups when compared to control values quoted in Chapter III.

4.3.3.4. Serial Studies

Serial studies were possible in 13 patients with CD. The results of C' and IC estimations on the sera from patients in active and quiescent phases of the disease are shown in Table 4.7.

Only 2 patients (GB and F) showed an increase in CH_{50} titre as the disease went into remission. In all other patients the CH_{50} titre increased as the disease became active.

Only one patient (F) showed a fall in ACA as the disease became quiescent. No relationship existed with change in disease activity and evidence of ICs.

C3 and C4 concentrations were higher in the active phase than in the quiescent phase in 5/9 and 6/9 patients, respectively.

Patient	Disease Status	CH ₅₀	ACA	C3 mg%	C4 mg%
LB	Active	32	16		
	Quiescent	32	2048		
GB	Active	32	-ve		
	Quiescent	128			
	Quiescent	256	32		
MC	Quiescent	16	32		
	Active	64	32		
CD	Active	64	-ve		
	Quiescent	32	-ve		
	Active		-ve		
TM	Quiescent	64	-ve	82	48
	Active	64	-ve	75	42
VF	Active	16	2	52	
	Quiescent	64	-ve	80	20.4
	Active	16	-ve	118	24
HI	Active	128	-ve	159	65
	Quiescent	16	-ve	109	55.5
DP	Quiescent	64	-ve	56	23.4
	Active	128	-ve	96	40.4
	Quiescent	64	-ve	64	22.8

contd.....

Table 4.7. contd.

Patient	Disease Status	CH ₅₀	ACA	C3	C4 mg%
CR	Active	32		95	43
	Quiescent	4		63	24
PT	Active	64	-ve	81	22
	Quiescent	32	-ve	94	28.2
BW	Active	128	-ve	90	34
	Quiescent	256	-ve	94	22.2
AW	Active	16	-ve	92	34
	Quiescent	128	-ve	89	32.6
GW	Quiescent			167	49.6
	Active			146	35.8

Table 4.7. (contd.) SERIAL STUDIES

CH₅₀, ACA, C3 and C4 estimations in
the sera of 13 patients with Crohn's
disease

4.4 DISCUSSION

32.2% of sera from patients with CD in this study were found to have evidence of circulating ICs. Serum components C3 and C4 were found to be significantly raised in patients with CD when compared to concentrations in serum from control subjects. An attempt was made to correlate these findings with length of history of disease, steroid therapy and disease activity. Results of CH_{50} , ACA and C3 and C4 determinations have been analysed accordingly.

4.4.1. DURATION OF DISEASE

Lack of correlation between length of history of disease and evidence of circulating ICs in patients with CD has been noted by several workers using a variety of methods of IC detection (Jewell and MacLennan, 1973; Hodgson, Potter and Jewell, 1977a). The results presented here are consistent with this. Estimation of CH_{50} showed no variation with length of disease process. ACA could not be correlated with duration of disease either. C3 and C4 concentrations were all elevated when compared to control values but there was no relationship with length of history of disease.

It appears that immunological activity does not vary with duration of CD. It is more likely that there would be fluctuations of immunological activity in the chronic relapsing course of the disease which may be revealed by serial studies of disease activity (see later).

Long term drug therapy, commonly used in the management of CD probably affects tests for immunological activity and may mask any clear relationships with duration of disease.

4.4.2. STEROID THERAPY

Corticosteroids are often used to control acute exacerbations of CD as well as in the management of patients with chronic unremitting disease.

Many clinical trials have been designed to study the clinical features of the disease in response to treatment with corticosteroids alone or in conjunction with AZA. The many side effects of steroid therapy e.g. Cushing's syndrome, often necessitate its withdrawal and continuation of treatment with other drugs, such AZA. AZA belongs to the class of drugs known as immunosuppressants, commonly used in cancer chemotherapy and in the prevention of transplantation rejection. Recently the mode of action of AZA has been suggested to be immunostimulatory (Gyte and Willoughby, 1977), possibly due to reduced suppressor cell activity (Hodgson, et al, 1978). AZA also has many unwanted side effects, including nausea, vomiting and bone marrow depression.

Evidence of the efficacy of both corticosteroids and AZA therapy in CD is controversial. Early papers (Brooke, Hoffman and Swarbrick, 1969; Avery-Jones, 1969; Drucker and Jeejeebhoy, 1970) reported AZA to be effective in the control of CD. Later this was disputed by Rhodes et al (1971). has been shown to be effective and the control of symptoms induced can be maintained by switching to AZA therapy (Willoughby et al, 1971; Rosenberg et al, 1976). The use of corticosteroids in the treatment of CD has recently been criticized (Smith et al, 1978).

Cortisone inhibits fibrous tissue formation and collagen deposition; whereas this does not happen with AZA (Drucker and Jeejeebhoy, 1970). Cortisone also causes lymphocytopenia, monocyto-

penia, eosinopenia and neutrophilia (Fauci and Dale, 1974; Yu et al, 1974) and decreased immunoglobulin production (Butler and Rossen, 1973). All sub-populations of lymphocytes are reduced in vivo by corticosteroids, particularly the thymus-derived T-cells (Fauci and Dale, 1974; Yu et al, 1974; Hodgson et al, 1978). Despite these observations very little is known about how corticosteroids affect the immune response. In tests for Ab-Ag detection no correlation has been found with steroid therapy: no correlation with inhibition of ADCC (Jewell and MacLennan, 1973): ACA (Hodgson, Potter and Jewell, 1977a) or Clq, C3, C4 or Factor B levels (Hodgson, Potter and Jewell, 1977b). CH₅₀ titre has been shown to rise during steroid and adrenocorticotrophic hormone (ACTH) therapy in UC (Fletcher, 1965).

In the present study no difference in the levels of CH₅₀, ACA, C3 and C4 was found in patients receiving steroid therapy and those who were not. The incidence of circulating ICs therefore does not appear to be affected by treatment with steroids.

Similar studies have been carried out on patients with UC by Neilsen et al (1976), and they suggested that corticosteroids may affect formation of ICs by causing a decrease in release of Ag from inflamed gut resulting in a decrease of IC production and consequently a decrease in C' activation. No such evidence of this was found in the studies on patients with CD.

4.4.2.1 Other Drugs

Although the mainstay of drug therapy in CD is the use of corticosteroids and AZA, a variety of other drugs are used in conjunction with them. A summary of other drugs used in the management of the patients included in this study was given in Table 4.5.

The effects of corticosteroids and AZA on immunological activity has already been discussed. Of the other drugs used in the management of CD, phenylbutazone being an anti-inflammatory agent, is the only one likely to have an effect on immunological activity.

4.4.3. DISEASE ACTIVITY

Many workers have attempted to correlate disease activity with evidence of circulating ICs and have shown that ICs appear to increase with severity of disease (Jewell and MacLennan, 1973; Teisberg and Gjone, 1975; Hodgson, Potter and Jewell, 1977a and 1977b). Most of these reports go into quite considerable clinical detail of parameters such as blood in faeces, number of bowel movements per day, signs of ulceration on sigmoidoscopy, cytological and biopsy findings, as described by de Dombal et al (1974). Such detail was not possible in this study and patients were described as having either quiescent or active disease according to their clinical notes.

Although 32.2% of sera from patients with CD studied were found to have excess ACA there was no difference in this between patients with active or quiescent disease. This does not agree with the results reported by Hodgson, Potter and Jewell (1977a) who found an increase in ACA with increase in severity of disease. There was also no difference in distribution of CH₅₀ titre and disease activity in the present study.

Comparison of C' components C3 and C4 revealed that both were raised in cases of active disease, though not significantly.

Teisberg and Gjone (1975) found no disturbances of C3 or C4 concentrations from normal and therefore no correlation with disease activity in CD. However, Hodgson, Potter and Jewell (1977b) found a tendency of C3 but not C4 levels to rise with increasing activity of disease. They found also raised Factor B levels in cases of active disease, thus implicating the alternative pathway of activation of the C' sequence in CD. Since both C3 and C4 levels were affected in this study the classical pathway is implicated.

4.4.3.1. Serial Studies

Serial studies on 13 individual patients underlined the group statistics that no change in evidence of ICs could be detected in active or quiescent phases of the disease. C3 and C4 concentrations were higher in over half of the patients in the active phase.

4.5 CONCLUSION

Although 32.2% of sera from patients with CD were found to have evidence of circulating ICs, this could not be correlated with duration of disease, steroid therapy or disease activity. The three parameters of the disease mentioned above are interrelated. CD of long duration is punctuated by acute exacerbations which require intense drug therapy. Since there was no difference between patients with active or quiescent disease it can be concluded that the disease is characterised by an ongoing immunological process.

C3 and C4 concentrations were elevated in all the parameters studied when compared to control values, but did not differ within the parameters, except in the case of active disease, when C3 and C4 levels were elevated when compared to quiescent disease, but not significantly so.

CHAPTER 5

EVIDENCE OF IN VIVO COMPLEMENT

ACTIVATION IN PATIENTS WITH

CROHN'S DISEASE

5.1 INTRODUCTION

Previous chapters have dealt with the measurement of complement (C') activity and serum factors C3 and C4. This gives a static picture of the C' system in immune complex (IC) diseases. The serum concentration of a protein is influenced by a number of variables; plasma volume, extra-vascular distribution, fractional catabolic rate and rate of synthesis (Hodgson, Potter and Jewell, 1977c). Studies of C3 metabolism give a more dynamic view of C' activation. C3 plays a key role in the C' activation sequence being involved in both the classical and alternative pathways. Of the C' components, C3 is the commonest C' protein, its serum concentration being 1200 mg% (Mayer, 1973). The next commonest is C4, with a serum concentration of 200 mg%.

Metabolic studies, using radiolabelled C3, have shown that although C3 levels were normal or raised in patients with Crohn's disease (CD), the fractional catabolic rate and synthesis were increased (Hodgson, Potter and Jewell, 1977c). Such turnover studies are complicated, difficult and time consuming, requiring medical supervision. Hence they were not possible in the present study.

A dynamic view of C' activation can also be gained by demonstration of C3 breakdown products in the sera of patients by immunoelectrophoresis (Laurell, 1965). This method has been used by Teisberg and Gjone (1973) to demonstrate the presence of C3 inactivation (C3i) products in the sera of patients with various forms of liver disease. Similar determinations have been made using sera from patients with systemic lupus erythematosus (SLE) and with rheumatoid arthritis (RA) (Versey, 1973; Versey, Hobbs and

Holt, 1973; Teisberg, 1975a) and from patients with inflammatory bowel disease (UC and CD) (Teisberg and Gjone, 1975; Neilsen et al 1978).

The principle of demonstration of C3 conversion in serum lies in the fact that during utilization of C', C3 is split into two fragments, C3a and C3b (Mayer, 1973). C3b becomes bound and participates in the C' sequence after which it is further degraded to C3c and C3d. These fragments, C3b, C3c and C3d, retain some of the antigenic characteristics of the parent molecule and will cross-react with anti-C3 antiserum but have a higher electrophoretic mobility and so will migrate faster than the native C3. Separation of C3i products can therefore be achieved by electrophoresis in one direction. Quantitation and visualisation can then be secondarily achieved by electrophoresis into Ab-containing agarose in a second dimension, at right angles to the first, when immuno-precipitation peaks form. It is thought that the converted peaks contain C3b, C3c and negligible amounts of C3d (Teisberg, 1975b).

In this study serum and plasma from patients with CD and an age and sex-matched population of control subjects were tested for evidence of C3i products. As the use of plasma or serum in the detection of C3i products is controversial (Versey, 1973; Teisberg, 1975b; Greenwood and Whittle, 1976; Pepys, 1977; Cumming and Verrier-Jones, 1977; Neilsen et al, 1978) it was initially decided to compare C3 conversion in plasma and serum from control subjects and patients with CD. In addition, C3 and C4 concentrations and ACA were measured in as many samples as possible, by the methods described in Chapter II. Nielsen et al (1978) showed a correlation between C3 activation and circulating ICs. The results of

detection of C3i products were examined for the existence of correlation with evidence of circulating ICs.

Evidence of circulating C3i products has been correlated with disease activity and prednisone medication in Goodpastures syndrome (Teisberg, 1975b) and disease activity in patients with CD (Teisberg and Gjone, 1975). The results presented here were examined for correlation with disease activity and steroid therapy.

5.2 PATIENTS, MATERIALS AND METHODS

5.2.1 PATIENTS

62 serum samples and 20 plasma samples from 51 patients with CD and 17 serum samples and 10 plasma samples from 27 control subjects were tested for evidence of C3i products. The age range of patients with CD was 18-76 years, mean 41.3 years. Control subjects were laboratory personnel and student volunteers. Their age range was 19-58 years, mean 26.3 years.

5.2.2 MATERIALS

Electrophoresis tank with cooling plate, U77 (after Kohn);
(Shandon Southern Instruments Ltd.);
Power pack V500/150 (Shandon Southern Instruments Ltd.);
Gel cutter and cutting blade (Shandon Southern Instruments Ltd.);
Glass plates 5x5 cm. (Kodak);
Water baths at 100°C and 56°C;
Hamilton microsyringe and adaptor (V.A. Howe);
Antiserum to C3 (Hoechst);
Agarose (BDH);
Barbitone buffer, pH 8.6, ionic strength 0.02 (Appendix I);
0.5 ml. serum/plasma sample;
Coomassie Brilliant Blue stain (BDH) (Appendix I);
Destaining solution (Appendix I).

5.2.3. METHODS

The method of two-dimensional immunoelectrophoresis adopted was a modified version of the one described by Laurell (1968) and similar to the automated method of Versey (1973). It was performed as described overleaf.

5.2.3.1. Preparation of agarose gel

The electrophoretic procedure takes place in two stages, first a simple electrophoresis in agarose gel, followed by a second at right angles to the first forcing separated serum proteins into agarose containing anti-C3 antiserum. Each stage requires slightly different preparation of the agarose gel.

Initially 4x3 ml. and 1x4 ml. of 1% agarose made up in barbitone buffer, pH 8.6, ionic strength 0.02, were melted in a water bath at 100°C and transferred to a second water bath at 56°C to equilibrate for 30 minutes.

For the first stage of electrophoresis, the plate was prepared as follows: 4 ml. of molten agarose were poured onto a level 5x5 cm. glass plate and allowed to solidify. Four wells were cut in the gel, 0.5 cm. from one edge. The gel was divided into four equal segments each containing a well. The samples were applied to the wells.

For the second stage, the segments of agarose were transferred to separate 5x5 cm. glass plates and the remainder of each plate filled with 3 ml. molten agarose containing 20 ul. of anti-C3 antiserum, which had been added to the molten agarose after equilibration at 56°C for 30 minutes.

5.2.3.2. Preparation of the Electrophoresis Tank and Power Pack

The electrode vessels were filled with barbitone buffer, pH 8.6, ionic strength 0.02. The prepared agarose plate was placed on a central cooling plate and connected to the buffer solution on either side by two wicks.

The power pack was used as a constant voltage supply. The apparatus was fitted with a circuit breaker which interrupted the current if the lid should be removed during electrophoresis. A polarity changer was used to change the current direction between successive runs and so prevent the creation of concentration gradients in the buffer compartments.

5.2.3.3. Serum Application and Electrophoresis

Serum samples were diluted 1:1 with saline and mixed gently. 2 μ l. were added to the wells in the first agarose plate, using a Hamilton microsyringe.

Electrophoresis was carried out in the first dimension at 350 volts for 45 minutes, causing separation of serum proteins towards the cathode.

Electrophoresis in the second dimension was performed at right angles to the first at 250 volts for 120 minutes. As the proteins migrated towards the cathode, C3 and its conversion products formed a precipitin line with the antiserum in the gel.

5.2.3.4. Staining

The precipitin peaks were visualised by staining with Coomassie Brilliant Blue. The agarose plate was first washed overnight in phosphate buffered saline (PBS) at 4°C to remove any unprecipitated proteins. Dehydration was effected by compression of the agarose, covered with filter paper and adsorbant tissue, with a 2 kg. weight. The agarose plate was stained in Coomassie Brilliant Blue for 5 minutes. Background stain was removed by washing in the dye solvent (destainer) for 15 minutes.

5.2.3.5. Controls

The position of the C3 peak was located by electrophoresis of commercial standard serum. The position of the C3i peak was located by electrophoresis of a serum sample, from a control subject, which had been left at room temperature for 7 days to allow natural conversion of C3.

The controls were run with each batch of test samples.

5.2.3.6. Blood Samples

Blood samples were collected from control subjects and patients with CD into plain bottles and allowed to clot at room temperature. Within 2 hours of collection the serum was spun off, dispensed into 0.5 ml. aliquots and stored immediately at -70°C .

For comparative experiments with EDTA plasma, blood samples were collected into EDTA tubes and centrifuged. The plasma was dispensed into 0.5 ml. aliquots and stored immediately at -70°C .

Freezing and thawing of samples was avoided, a fresh aliquot being thawed once only prior to use.

5.2.4. C3 AND C4 DETERMINATIONS

C3 and C4 concentrations in samples tested for C3 conversion were measured by the method of single radial immunodiffusion described in Chapter II.

5.2.5. ANTICOMPLEMENTARY ACTIVITY

ACA titre in samples tested for C3 conversion was estimated by the method described in Chapter II.

5.2.6. DISEASE ACTIVITY

The patients with CD were divided into groups of active and quiescent disease according to the criteria described in Chapter 4.

5.2.7. STEROID THERAPY

The patients with CD were divided into 2 groups according to whether they were receiving steroid therapy or not.

5.2.8. STATISTICS

The χ^2 test was used to compare evidence of C3i products in plasma and serum; in control subjects and patients with CD; in patients with active and quiescent disease and in relation to steroid therapy.

The students t-test was used to determine whether C3 and C4 concentrations differed in samples showing evidence of C3i products and in those that did not.

The Kolmogorov-Smirnov χ^2 test (Seigel) was used to compare ACA in samples showing C3i products and in those that did not.

5.3. RESULTS

The results of two-dimensional electrophoresis to demonstrate the presence of C3i products in sera and plasma samples from control subjects and patients with CD are presented as follows.

5.3.1. CONTROL SUBJECTS

Evidence of C3 conversion was not found in any of the serum or plasma samples from control subjects.

5.3.2. PATIENTS WITH CROHN'S DISEASE

Evidence of C3 conversion was found in 20/62 serum samples and 7/20 plasma samples from patients with CD. There was no significant difference between evidence of C3i products in serum or plasma ($\chi^2 = 0.4$, $p > 0.5$).

Taken together, 32.9% of serum and plasma samples from patients with CD showed evidence of C3 conversion. This was significantly different to the control group ($\chi^2 = 11.82$, $p < 0.01$).

5.3.3. C3 AND C4 CONCENTRATION

C3 and C4 concentration are expressed in mg% of serum or plasma.

Table 5.1. shows the mean C3 and C4 concentrations \pm standard deviation (SD) in sera and plasma from control subjects and from patients with CD both showing C3i products and not showing C3i products.

Statistical analysis revealed that C3 levels were significantly raised in samples from patients with CD, whether they showed C3 conversion or not ($t = 4.94$, $p < 0.005$ and $t = 5.38$, $p < 0.005$, respectively). C4 levels were significantly raised in samples from

Patient Group	C3 mg% \pm 1 S.D.		C4 mg% \pm 1 S.D.	
Control subjects	68.62 \pm 14.98 (21*)		28.88 \pm 14.15 (21)	
Patients with CD C3i +ve	102.32 \pm 30.26 (25)		30.34 \pm 9.33 (25)	
Patients with CD C3i -ve	100.36 \pm 35.79 (55)		36.76 \pm 13.55 (53)	
Difference from Control Mean	t	p	t	p
C3i +ve	4.94	<0.005	0.412	>0.25
C3i -ve	5.38	<0.005	2.41	<0.01

Table 5.1. Mean C3 and C4 concentrations in control subjects and patients with Crohn's disease with and without evidence of C3 conversion, with significance of difference from control mean

* = Number of samples

patients with CD who had no evidence of C3 conversion ($t = 2.41$, $p < 0.01$). There was no difference in C4 levels in samples from patients with CD who had circulating C3i products when compared to control values ($t = 0.41$, $p > 0.25$).

When C3 levels were examined for variation in samples from patients with CD, no significant difference was found whether they showed C3 conversion or not ($t = 0.26$, $p > 0.25$). In contrast, C4 levels were significantly lower in samples from patients with CD showing C3 conversion ($t = 2.44$, $p < 0.01$).

5.3.4. ANTICOMPLEMENTARY ACTIVITY

ACA has been defined in Chapter II.

12/26 samples from patients with CD that showed C3i products were found to have raised ACA (46%).

10/54 samples from patients with CD that showed no conversion of C3 were found to have raised ACA (18.5%).

Statistical analysis of the cumulative ACA distributions revealed this difference to be significant ($\chi^2 = 6.83$, $p < 0.05$).

5.3.5. DISEASE ACTIVITY

6/25 samples that showed C3i products were from patients in the active phase of the disease (24%).

14/52 samples that showed no evidence of C3 conversion were from patients in the active phase of the disease (26%).

Statistical analysis revealed no relationship between conversion of C3 and disease activity ($\chi^2 = 0.076$, $p > 0.95$).

5.3.6 STEROID THERAPY

14/27 samples that showed C3 conversion were from patients receiving steroid therapy (51.8%).

36/55 samples that showed no evidence of C3 conversion were from patients receiving steroid therapy (65.5%).

Statistical analysis revealed no relationship between conversion of C3 and steroid therapy ($\chi^2 = 1.4$, $p > 0.5$).

Results of ACA, disease activity and steroid therapy in relation to evidence of C3 conversion in samples from patients with CD are summarised in Table 5.2.

Parameter measured	C3i +ve	C3i -ve
ACA +ve	12*	10
ACA -ve	14	44
$\chi^2 = 6.83 \quad p < 0.05$		
Active Disease	6	14
Quiescent Disease	19	38
$\chi^2 = 0.076 \quad p > 0.95$		
Steroid Therapy	14	36
No steroid Therapy	13	19
$\chi^2 = 1.4 \quad p < 0.5$		

Table 5.2. Evidence of C3 conversion in relation to
ACA, disease activity and steroid therapy

* = number of samples tested.

5.4. DISCUSSION

In vivo activation of C' component C3 as revealed by crossed immunoelectrophoresis was found in 32.9% of serum and plasma samples from patients with CD. This percentage is lower than the figures of 52.4% and 5.17% reported by Teisberg (1975b) and Teisberg and Gjone (1975), respectively. These workers studied C3 conversion in serum samples only. If the results presented here are considered separately, the percentage of serum samples showing C3 conversion is 32.3%, whilst the percentage of plasma samples is 35%. However, there was no significant difference between the incidence of C3i products in serum or plasma.

Conversion of C3 was not found in any of the serum or plasma samples tested from control subjects. This indicates that in vitro conversion of C3 does not occur under the conditions of these experiments. Demonstration of C3i products in sera and plasma from patients with CD can therefore, be taken as evidence of in vivo activation of the C' sequence.

There is much controversy as to whether serum or EDTA plasma should be used in experiments to demonstrate C3i products by immunoelectrophoresis. Many workers advocate using EDTA plasma (Versey, 1973; Versey, Hobbs and Holt, 1973; Greenwood and Whittle, 1976; Neilsen et al, 1978). It is thought that the coagulation process accelerates in vitro conversion of C3 (Krøll, 1970) but this has been disputed (Teisberg, 1975b). Providing serum samples are stored at -75°C or used within 2 hours of collection, in vitro conversion of C3 does not occur to any appreciable extent (Laurell and Lundh, 1967; Teisberg and Gjone, 1973; Teisberg et al, 1973; Teisberg, 1975a). Laurell and Lundh (1967) did report traces of

C3i products in sera from control subjects handled in the manner described. This was not substantiated in the results presented here. Serum and plasma samples used in these experiments were handled carefully, being stored at -70°C within 2 hours of collection and thawed once only prior to use. Because of the lability of C3 in vitro a rapid method of high voltage immunoelectrophoresis is desirable (Teisberg, 1975b), such as the one described here. A cooling plate was used during the electrophoretic procedure to help minimise in vitro conversion of C3. In addition, it has been suggested that plasma may be unreliable in immunoelectrophoresis, as fibrin may precipitate in calcium-containing gels (Teisberg, 1975a and 1975b). This was unlikely to have affected experiments described here, as the barbitone buffer used to make up the agarose contained no calcium. More reliable results may be produced if EDTA is incorporated in the making up of the agarose when serum is to be used (Neilsen et al, 1978). However, the conditions of the experiments were considered to be reliable since no C3i products were detected in serum or plasma samples from control subjects.

C' components C3 and C4 were measured in most of the samples tested for C3i products. Versey (1973) had observed C3 and C4 conversion in plasma from patients with SLE and RA with low levels of C3 in SLE and raised C3 levels in RA. Further studies reported by Versey, Hobbs and Holt (1973) included patients with a variety of inflammatory and neoplastic diseases. They found raised C3 and normal C4 levels in these patients but both groups gave evidence of conversion products. Similar measurements on sera from patients with SLE (Teisberg, 1975b) and with inflammatory bowel disease (Teisberg and Gjone, 1975) showed C3 conversion products in some patients

with SLE with abnormally low levels of C3 and in patients with UC and CD with near normal C3 and C4 levels. The patients with CD in this study had raised levels of C3 when compared to control values whether C3 conversion was demonstrated or not. C4 levels were normal in patients showing C3 conversion and raised in patients with no evidence of C3 conversion. Raised serum C3 and the presence of C3i products reflects an increase in synthesis and catabolism i.e. an increased turnover rate (Hodgson, Potter and Jewell, 1977c). This indicates activation of the C' sequence possibly by ICs in the inflamed mucosa of the bowel (Ballard and Shiner, 1974). Levels of C4 within the normal range may indicate that the C' system is being activated via the alternative pathway, possibly by bacterial endotoxins or proteolytic enzymes in the damaged mucosa (Hodgson, Potter and Jewell, 1977c). However, the classical pathway may be involved since both C3 and C4 levels have been shown to be raised in patients with CD (Chapter III) and that there is a positive correlation between them, although raised C3 and C4 levels may be a reflection of the disease process causing an increase in the cells that manufacture these proteins. Activation of the C' system via the classical pathway would involve the consumption of C4 resulting in lowered concentrations of this protein. If the rate of synthesis is not increased as it is with C3 synthesis, then C4 levels would remain low. Kinetic studies such as those of Hodgson, Potter and Jewell (1977c) would reveal whether the catabolic rate and synthesis of C4 are altered in patients with CD.

Neilsen et al (1978) attempted to correlate the demonstration of C3i products with the presence of ICs in the sera of patients with UC. They found evidence of C3i products in both IC positive and IC negative sera but conversion of C3 was significantly greater in the

IC positive group. Similarly in the present study, evidence of ICs, as shown by measurement of ACA, was found in 46% of samples from patients with CD that showed C3 conversion and in 18.5% of samples which did not. The difference was significant, thus suggesting that ICs are the cause of C3 activation in patients with CD.

Teisberg and Gjone (1975) found a positive correlation between disease activity and evidence of C3i products in the serum of patients with CD. No such correlation was found here. 24% of samples that had evidence of circulating C3i products and 26% of samples that showed no evidence of C3 conversion were from patients with active disease.

Fewer patients on steroid therapy had evidence of C3 activation (51.8%) than those who showed no evidence of C3 activation (65.5%) but the difference was not significant.

5.5. CONCLUSION

C3i products were demonstrated in 32.9% of serum and plasma samples from patients with CD. This was taken to be evidence of in vivo activation of C3 and the C' system. Activation of the C' system was thought to be caused by circulating ICs since their presence could be correlated to C3 activation.

No relationship was found between C3 activation and disease activity or steroid therapy.

C3 levels were raised whether C3 conversion was demonstrated or not. C4 levels were raised in samples showing no conversion of C3 but normal in samples showing C3 conversion. It was postulated that C3 and C4 metabolism and synthesis may be altered in patients with CD and that kinetic studies may reveal this.

CHAPTER 6

GEL FILTRATION STUDIES ON ANTICOMPLEMENTARY
ACTIVITY POSITIVE SERA FROM PATIENTS WITH
CROHN'S DISEASE.

6.1. INTRODUCTION

The presence of circulating immune complexes (IC) in some of the patients with Crohn's disease (CD) has been indicated by the demonstration of higher than normal anticomplementary activity (ACA) in the sera of these patients (Chapter III).

Measurement of ACA detects ICs of less than 11S in size (Hodgson, Potter and Jewell, 1977a). In order to further characterize ICs in the sera of patients with CD in terms of molecular weight (MW) and immunoglobulin (Ig) content, it was decided to fractionate ACA positive sera by column chromatography. This work is described in this chapter.

The material chosen for fractionation of serum was the molecular sieve Sepharose 6B.

According to the theory of molecular sieving, proteins may be separated by virtue of their molecular dimensions. In a gel such as Sepharose 6B, an equilibrium is maintained between the liquid phase around the gel particles and the gel phase. Depending on the pore size of the gel, molecules may diffuse from the liquid phase to the gel phase. The rate at which a molecule moves down a column depends on the number of beads entered. Because the solute molecules maintain a constant equilibrium between gel and liquid phase a sample moves down a column as a band. Large molecules, above the exclusion limits of the gel, will not enter the beads and so move with the advancing solute front while smaller molecules enter the beads and must traverse this space as well as the volume around the beads. All sizes of molecules above the exclusion limits of the gel appear in the void volume and are not separated from each other. Smaller molecules of related groups e.g. Igs will leave the column

in order of decreasing size. MW is not the only factor governing the entry of molecules into a gel. Molecular shape and hydration are important. Hence proteins and carbohydrates have different exclusion limits in terms of MW.

Sephacrose 6B has the following characteristics, which are necessary for the separation of IgG, IgA and IgM.

1. The approximate exclusion limit for proteins is 4×10^6 daltons. The MW of IgG is 1.6×10^5 ; IgA is 1.7×10^5 and IgM is 9×10^5 , which are all below the exclusion limits of Sepharose 6B.
2. The correct pore size. The proteins to be separated must be able to diffuse into the beads for molecular sieving to occur.
3. The gel is inert in relation to the material to be separated. Charged groups on the gel or affinity for the material will interfere with size separation.
4. The gel is finely dispersed, thus allowing rapid diffusion and effective separation, but not so fine as to slow the flow of solute molecules. Beads give good flow characteristics.

15 sera from 13 patients with CD included in the study described in Chapter III, which had positive ACA, were fractionated on a column of Sepharose 6B. Three pooled fractions were analysed for ACA, protein, IgG, IgA and IgM.

6.2. PATIENTS, MATERIALS AND METHODS

6.2.1. PATIENTS

13 patients with CD, described in Chapter III, were included in this investigation. There were 8 males and 5 females, age range 21-76 years, mean age 41.8 years.

6.2.2. COLUMN CHROMATOGRAPHY

6.2.2.1. Materials

Sepharose 6B (Pharmacia);
100x25 cm column (Pharmacia);
10/150 buffer (Appendix I);
combi coldrac 5201 cabinet (LKB);
Biocal chart recorder (LKB);
Bromma 7000 ultroac fraction collector (LKB);
UV spectrophotometer SP 600 (Unicam);
Diaflo PM 10 membrane and Amicon filter (Amicon).

6.2.2.2. Serum samples

Venous blood samples were collected into plain bottles and allowed to clot at room temperature. Within 2 hours of collection the serum was spun off, dispensed into 0.5 ml. aliquots and stored immediately at -70°C . 2 aliquots were required to give 1 ml. of sample which was diluted 1 in 3 with 10/150 buffer before application to the column.

6.2.2.3. Method

Sepharose 6B was obtained preswollen and a 100x2.5 cm. column poured. The column was equilibrated with 10/150 buffer and run at 8 ml./hour at 4°C . The void volume was estimated using blue dextran in 10/150 buffer.

The sample was layered on to the column. 4 ml. fractions were collected on an LKB ultrorac overnight. A 280 nm. profile was recorded on an LKB Biocal chart recorder at speed 10 mm./hour.

The column had previously been calibrated to give 3 major fractions with MW ranges of $>10^6$; $2 \times 10^5 - 10^6$; $5 \times 10^4 - 2 \times 10^5$. When all the protein had been eluted, the 4 ml. fractions were pooled to give these 3 major fractions. The 3 major fractions were concentrated using an Amicon filter and Diaflo PM10 membrane, nominal exclusion weight 10^4 daltons, at 0°C . The first fraction (I) was concentrated from 16 ml. to 1 ml. and the second (II) and third (III) from 28 ml. and 24 ml., respectively to 2 ml. The concentrates were divided into 0.5 ml. aliquots and snap frozen in liquid nitrogen until required for protein, Ig and ACA assay.

6.2.3. FOLIN-CIOCALTEAU PROTEIN ESTIMATION

6.2.3.1. Materials

Solution A 5% copper sulphate;

B 10% sodium potassium tartrate;

C 2% sodium bicarbonate in 0.1 M sodium
hydroxide;

D 1 volume Folin-Ciocalteu reagent +
2 volumes distilled water;

E 1 ml. A + 1ml. B + 8 ml. distilled water;

F 50 ml. C + 1 ml. E.

UV spectrophotometer SP 600 (Unicam);

Serum fractions I, II and III.

6.2.3.2. Serum fractions

Prior to protein estimation, fraction I was diluted 1 in 5; fraction II, 1 in 5 or 1 in 30 and fraction III, 1 in 100, 1 in 200 or 1 in 600, depending on the protein content, with 10/150 buffer.

6.2.3.3. Method

0.4 ml. of sample were added to 4 ml. solution F and 0.4 ml. of solution D. The solutions were whirlmixed together and the colour allowed to develop in the dark for 30 minutes at the ambient temperature. Each sample was estimated in duplicate. The optical density was read at 650 nm. against a reagent blank of 10/150 buffer and solutions F and D.

A calibration curve was plotted from known concentrations of bovine serum albumin dissolved in 10/150 buffer and assayed following the above procedure.

6.2.4. ESTIMATION OF IgG, IgA and IgM CONCENTRATION BY SINGLE RADIAL IMMUNODIFFUSION

6.2.4.1. Materials

Borate buffer, pH 8.6, ionic strength 0.05 (Appendix I);
I.D. agar tablets (Oxoid);
Antisera to IgG, IgA and IgM (Wellcome);
Ig standards for IgG, IgA and IgM (Hoechst);
Hamilton microsyringe and adaptor (V.A. Howe);
Water baths at 100°C and 56°C;
Phosphate buffered saline (PBS) pH 7.35±0.05 (Appendix I);
Damp chamber at 4°C;
Well cutter (Wellcome);
Coomassie Brilliant Blue stain (BDH) (Appendix I);
Destaining solution (Appendix I).

6.2.4.2. Serum fractions

With the exception of fraction III, serum fractions were diluted 1 in 2 with PBS before application to the IgG plate. Fraction III was applied undiluted to the IgG plate as were all fractions to the IgA and IgM plates.

6.2.4.3. Method

5 ml. plates of 1% agar containing the appropriate concentration of antiserum (IgG, 1.5%; IgA, 2.5%; IgM, 1.2%) were prepared, using the method described for measurement of C3 and C4 (Chapter II). The molten agar was allowed to equilibrate at 56°C for 30 minutes and the antiserum was then added. The agar was allowed to solidify and dry at room temperature. Wells were cut in the agar using a gel cutter and template.

2 μ l. of sample were applied to each well. There were 9 samples and 3 standards to each plate. The concentrations of the standards were:

IgG; 38, 90 and 185 mg%;

IgA; 90, 168 and 325 mg%;

IgM; 62, 163 and 258 mg%.

Diffusion was allowed to take place over 72 hours in a damp chamber at 4°C.

The plates were dehydrated and dried at room temperature before the diffusion rings were visualised by staining in Coomassie Brilliant Blue for 5 minutes. Background stain was removed by washing in the destaining solution.

Diameter² (D^2) was plotted against concentration.

6.2.5. ANTICOMPLEMENTARY ACTIVITY

The method of estimation of ACA has been described fully in Chapter II. The method was applied to determinations in the 3 serum fractions obtained from Sepharose 6B fractionation of 15 serum samples from 13 patients with CD.

6.3. RESULTS

Results of protein, IgG, IgA and IgM measurements and estimation of ACA titre in the 3 fraction pools obtained from fractionation of 15 serum samples from 13 patients with CD are shown in Table 6.1. The protein and Ig concentrations are expressed in mg% of concentrate.

9/15 Fraction I pools were found to contain IgG; 3/15 were found to contain IgA and one was found to contain IgM. ACA was detected in all fraction pools that contained IgG and in one that contained IgA but no IgG.

All 15 Fraction II pools were found to contain IgG and IgM and 6/15 were found to contain IgA. ACA was detected in 11/15 fraction pools.

All 15 Fraction III pools were found to contain IgG and 13/15 were found to contain IgA. IgM was detected in 2 fraction pools. ACA was not detected in any of the fraction pools.

The highest protein concentration was found in Fraction III in all cases, the next highest in Fraction II and the lowest in Fraction I. IgG followed a similar distribution. IgA was almost equally distributed in Fraction II and III. IgM was predominantly found in Fraction II. In each of the 9 cases containing IgG in Fraction I the concentration of IgG represented a low percentage of the total IgG found in all 3 Fraction pools, i.e. 13.5%; 7.1%; 4.6%; 3.6%; 1.3%; 23.2%; 2.3%; 2.3%; and 17%, respectively. In each of the 3 cases containing IgA in Fraction I the concentration of IgA represented a high percentage of the total IgA, i.e. 55.8%; 16.4% and 20%, respectively.

Patient	Protein	Fraction I				Fraction II				Fraction III					
		IgG	IgA	IgM	ACA Titre	Protein	IgG	IgA	IgM	ACA Titre	Protein	IgG	IgA	IgM	ACA Titre
1. HW	85**	55			64	357	14		4		4705	340	56		
2. MC	52.2					277.5	10		26	32	7800	346	156		
3. GH	18.7					502.5	138	196	26		6600	368	32		
4. LB	41.2	25			8	470.2	34		4	8	3975	294	72		
5. HW	50					1212	88	82	24	16	8560	720	56		
6. SA	45	16		4	4	1065	112	82	26	8	3800	218			
7. LB	30	16			2	2182	180	28	26	2	3875	248		2	
8. DC	30	4			2	1375	112		26	4	2600	196	66		
9. SC	7.5					862.5	128	10	16		4250	208	56		
10. BB	3.75					322.5	68	4	40	16	825	68	62		
11. TM	75	58			4	532.5	72		12	4	2175	120	94		
12. TK	23.7	14			16	585	64		4	8	5500	528	82		
13. JR	153.7		134		8	2300	304		58		12750	548	106		
14. VF	38.7	6	12		4	937.5	116		12	8	2700	144	48		
15. LP	107.5	46	22		1024	160	112		24	2	2475	112	112	2	

Table 6.1. Protein, IgG, IgA and IgM concentrations (mg%) and ACA titre measured in Fraction I, II, and III

obtained from fractionation of 15 sera from 13 patients with Crohn's disease on Sepharose 6B

** = Protein, IgG, IgA and IgM expressed in mg%.

Figure 6.1. shows the elution profile of a reference solution of IgG, IgM and albumin in 10/150 buffer. IgG and IgA were detected in the fraction pools as shown.

Figures 6.2. to 6.6. show elution profiles of sera from 5 patients. The figures demonstrate the distribution of protein, IgG, IgA, IgM and ACA.

Figure 6.1. Elution profile of a reference solution containing
60 mg% IgM, 400 mg% IgG and 400 mg% human serum
albumin in 10/150 mM buffer.
Absorbance measured at 280 n.m.

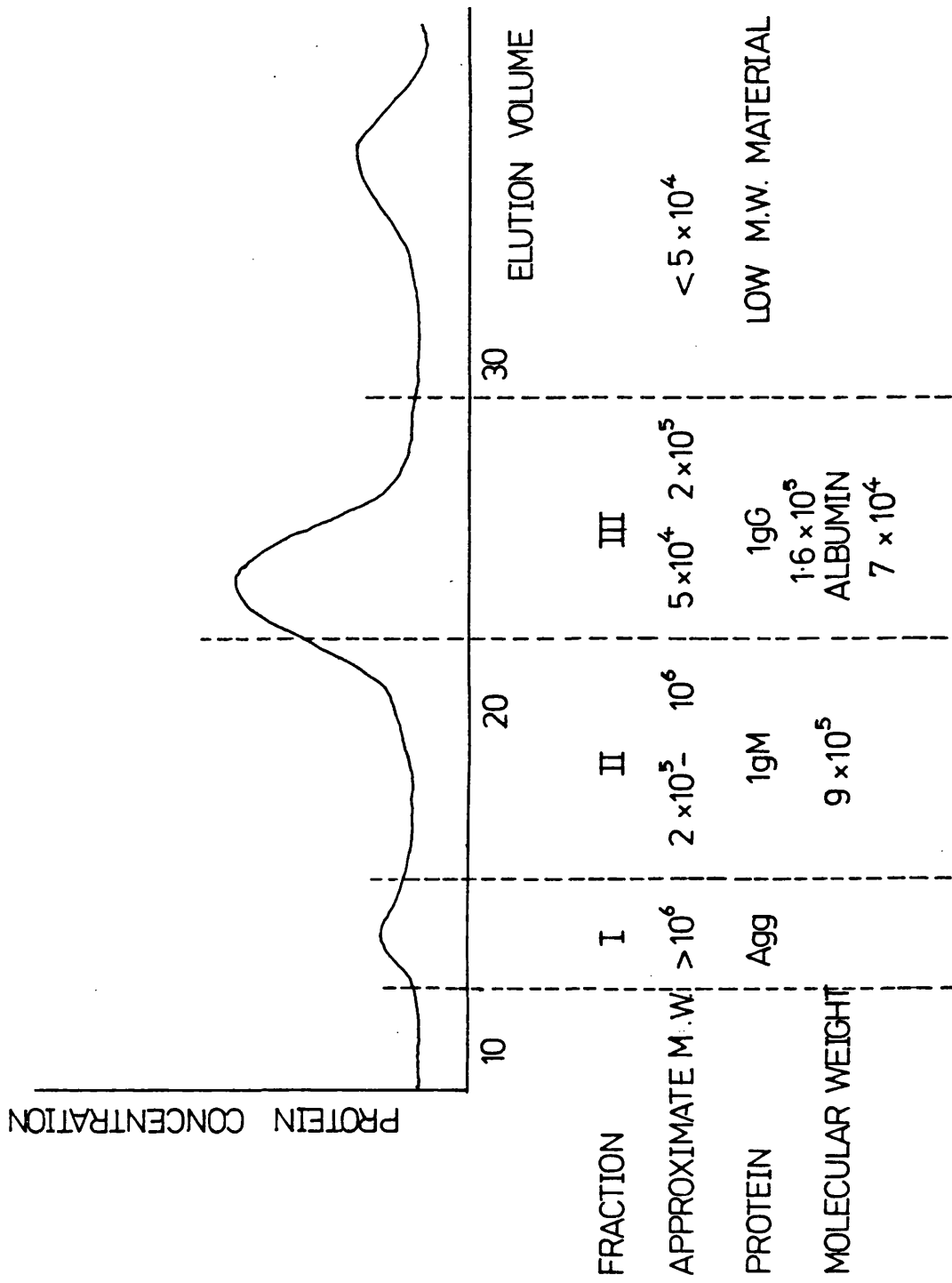


FIGURE 6.1

Figure 6.2. Elution profile of serum from patient VF fractionated
on Sepharose 6B column.

Absorbance measured at 280 nm.

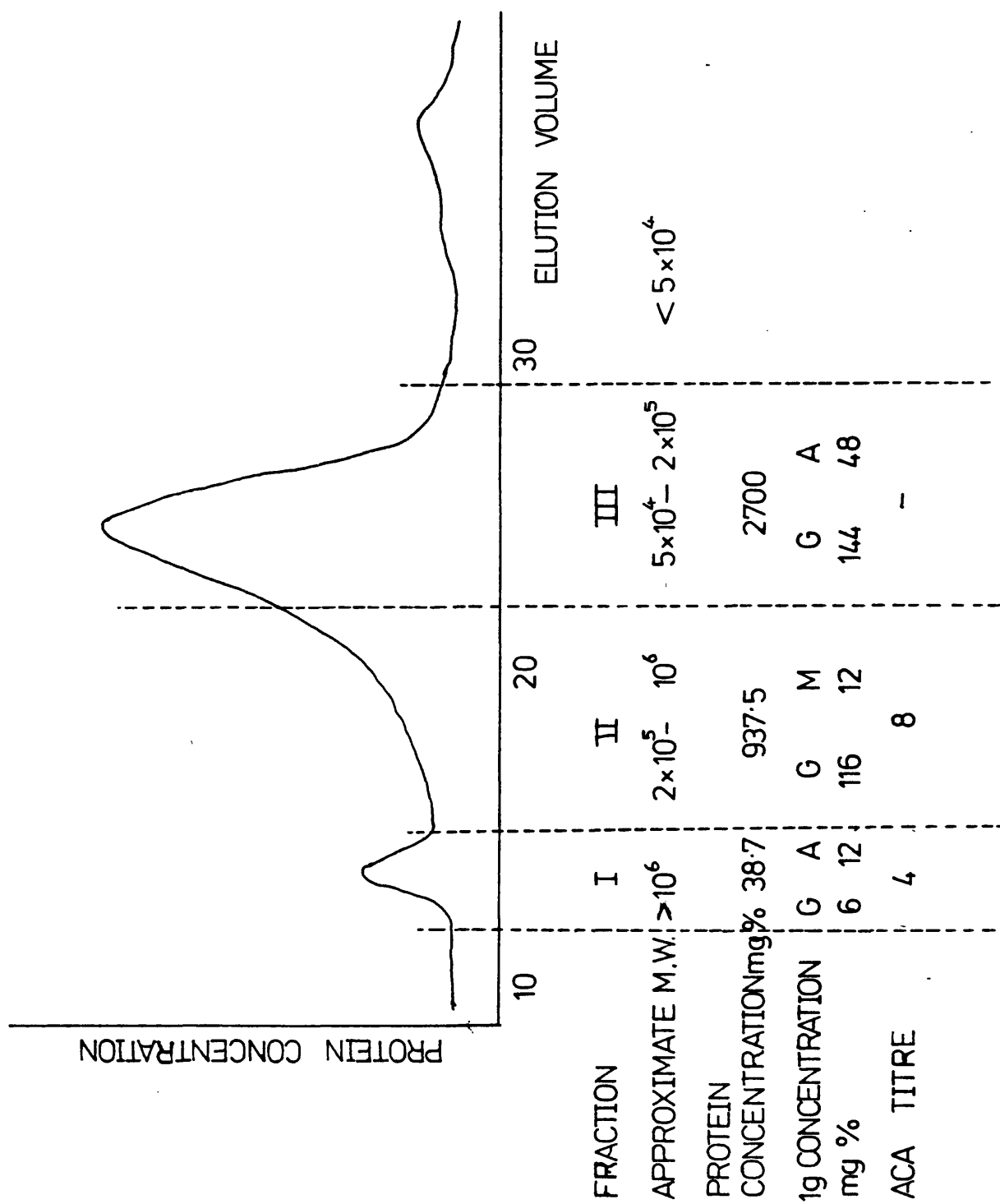


FIGURE 6.2

Figure 6.3. Elution Profile of serum from patient SA fractionated
on Sepharose 6B column.
Absorbance measured at 280 nm.

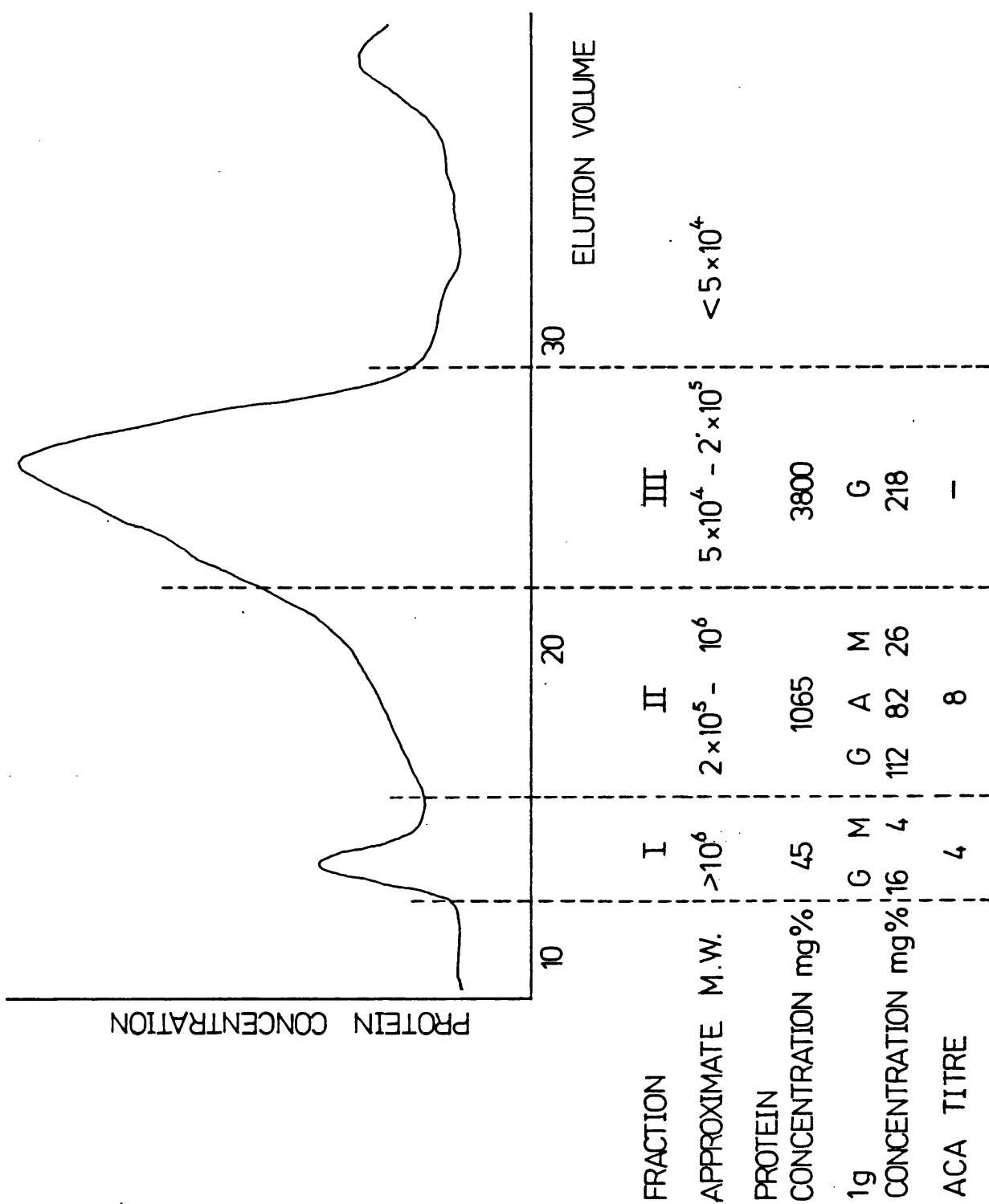


FIGURE 6.3

Figure 6.4. Elution profile of serum from patient JR fractionated on Sepharose 6B column.
Absorbance measured at 280 nm.

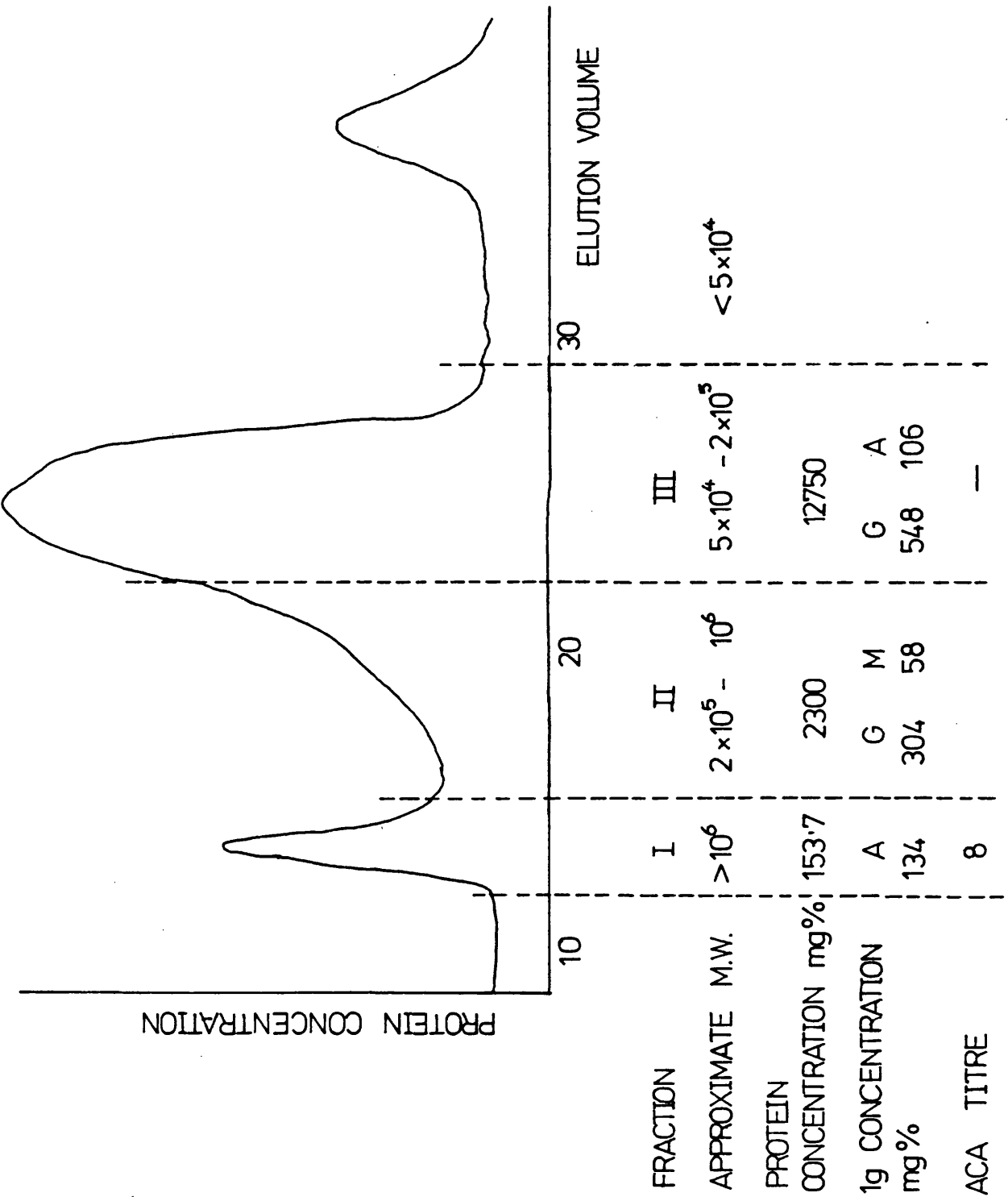


FIGURE 6-4

Figure 6.5. Elution profile of serum from patient MC fractionated
on Sepharose 6B column.
Absorbance measured at 280 nm.

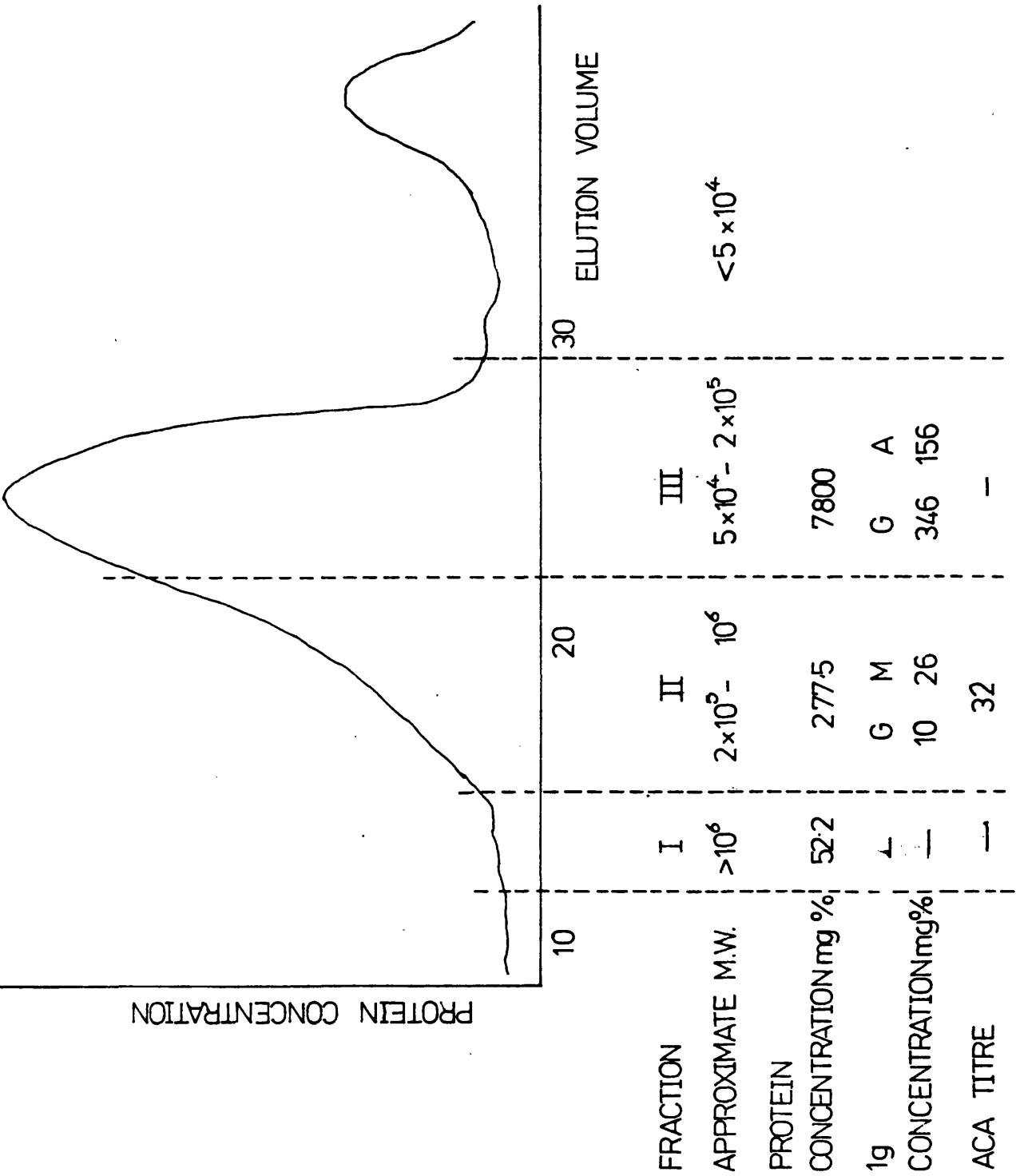


FIGURE 6.5

Figure 6.6. Elution profile of serum from patient DC fractionated
on Sepharose 6B column.
Absorbance measured at 280 nm.

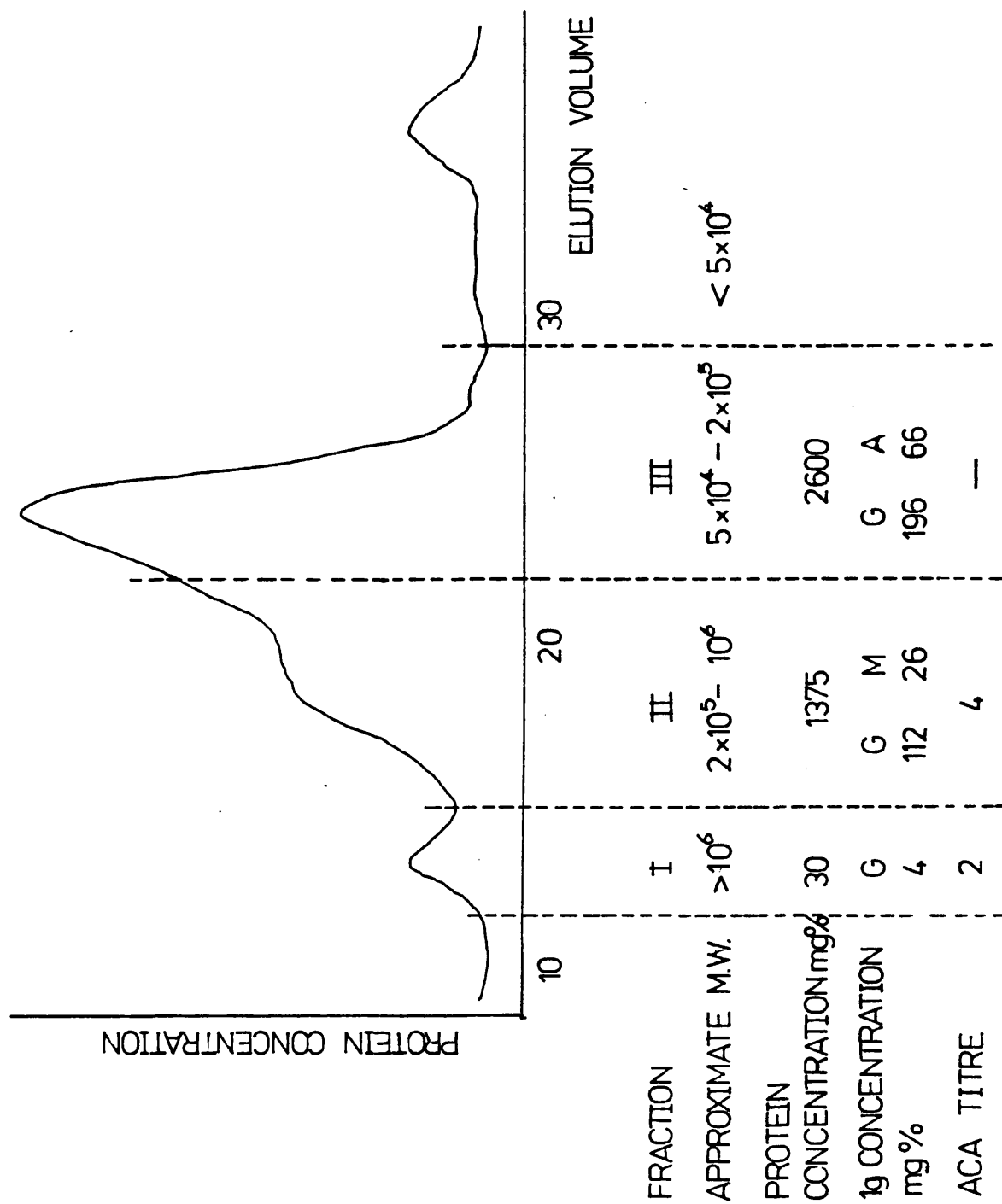


FIGURE 6.6

6.4. DISCUSSION

Gel filtration of ACA positive sera from patients with CD on a column of Sepharose 6B showed that the ACA was confined to Fraction pools I and II. The MW range of these 2 fractions is $>10^6$ and $10^6-2 \times 10^5$ daltons, respectively. This is above the MW of monomeric IgG and IgA, but not IgM. Measurement of Ig classes by immunodiffusion revealed IgG and IgA in some of the Fraction I and II pools. IgG found in Fraction I was thought to be part of ICs rather than aggregates because the amount of IgG detected in these fractions was a small percentage of the total IgG measured in all 3 fraction pools. It has been shown that for IgG aggregates to fix C' they must be present in a high concentration (Nielsen and Svehag, 1976). IgG detected in Fraction II pools was probably in the form of dimers or small complexes. Again, the amount of IgG found in Fraction II represented a small percentage of the total. A high percentage of total IgA, however, was found in Fraction I and II pools and was probably in the form of aggregates or dimers, possibly as secretory IgA. It has been shown that serum IgA levels may be raised in patients with CD and that some of it may be secretory IgA (Deodhar et al, 1969; Perrett et al, 1971).

As expected IgM was found in all 15 Fraction II pools. This is in accord with its MW which falls within the MW range of Fraction II, i.e. between 10^6 and 2×10^5 . One Fraction I sample was found to contain IgM. It is possible that it could be present here as a component of a complex.

IgG was found in high concentration in all Fraction III pools where it must be presumed to be in its monomeric form. IgA was

found in 13/15 Fraction III pools and would also be in its monomeric form. Two Fraction III samples also contained traces of IgM and it must be assumed that some degradation had taken place for IgM to be detected in a fraction of MW range lower than its own. ACA was not detected in any of the Fraction III pools.

Protein concentration was found to decrease through Fraction III to II to I. This distribution was similar to that of IgG.

Detection of ICs by ACA estimation depends on activation of the C' system. The C' system can be activated by complexes containing IgG via Clq fixation in the classical sequence and by complexes containing IgA via properdin in the alternative sequence.

It can be concluded from this work that ICs detected in the sera of patients with CD may contain IgG or IgA or possibly both. IgM can also activate the C' system when complexed with Ag and it is possible that IgM complexes may also be involved in patients with CD since one serum sample was found to have IgM in the high MW Fraction I.

Serum fractionation by column chromatography has long been used to characterise complexes in the sera of patients with a variety of diseases. In 1967, Soothill and Hendrickse reported complexes containing C' component C3 in the high MW fraction after separation of sera from children with a nephrotic syndrome on a column of Sephadex G-200. Since the MW of C3 is 1.85×10^5 daltons, it would be expected to appear in the same fraction as monomeric IgG or IgA. That it was found in a high MW fraction indicates that it was present in a complexed state.

ICs have been detected in the serum of patients with other inflammatory diseases such as rheumatoid arthritis (RA), ankylosing spondylitis (AS) and systemic lupus erythematosus (SLE) by a variety of methods including the inhibition of antibody-dependent cytotoxic reactions (MacLennan, Loewi and Howard, 1969; Corrigan et al, 1978), platelet aggregation (Norberg, 1974) and inhibition of Fc rosette formation (Morito et al, 1976). Gel filtration of the sera in the afore-mentioned publications on columns of Sephadex G-200, Sephadex G-150 and Sepharose 6B and 4B showed that ICs were found in high MW fractions and that they contained IgG which was not normally found in these fractions. Plasma from patients with RA have been shown to contain IgG which on Sepharose 6B analysis was found to be in the fractions between the IgG and IgM markers (Pope et al, 1974). ICs have been detected in the sera of patients with dermatitis herpetiformis (Mowbray et al, 1973). by methods of ACA titration and Clq precipitation. Subsequent fractionation of these sera revealed ACA in high MW fractions containing IgG.

Similar studies have been carried out on sera from patients with inflammatory bowel disease. Evidence of ICs in patients with CD and UC was indicated by the ability of their sera to inhibit antibody-dependent cytotoxic reactions (Jewell and MacLennan, 1973). After fractionation on a column of Sepharose 6B, inhibitory activity was found in fractions with MW greater than that of IgG but lower than that of IgM. Similar findings were reported by Henderson et al (1976). In addition, Hodgson, Potter and Jewell (1977a) demonstrated the presence of ICs in the sera of patients with CD by ACA assay and on Sepharose 6B separation found peak ACA in high MW fractions containing IgG.

The results reported in this chapter are consistent with the published results mentioned above, except that none of the authors described detection of IgA in high MW fractions. In the present study IgA was often found in fractions with a MW range above its own monomeric MW. Although high MW IgA is likely to be in the form of aggregates or secretory IgA, the possibility of IgA being involved in complexes cannot be discounted. It was concluded therefore, that ICs detected in the sera of patients with CD in this study were found to contain high MW IgG and possibly IgA.

Other analytical methods are available for further characterisation of ICs, such as sucrose density separation (Mowbray et al, 1973; Norberg, 1974; Henderson et al, 1976), diethylaminoethyl-cellulose separation (MacLennan, Loewi and Howard, 1969; Henderson et al, 1976) and ultracentrifugation (Pope et al, 1974; Morito et al, 1976). Further information regarding the nature of the ICs found in the sera of patients with CD in this study might be gained from the application of such analytical methods. Time did not allow this in the present study.

6.5. CONCLUSION

ICs have been detected in 32.2% of sera from patients with CD by estimation of ACA. On fractionation of serum samples with evidence of ICs the ACA was found to be confined to two fraction pools of MW range $>10^6$ and $2 \times 10^5 - 10^6$ daltons. IgG and IgA were measured in these fractions by immunodiffusion. As the MW of IgG and IgA are lower than the MW range of these fractions it was concluded that the ICs were composed of IgG and possibly IgA complexed with antigen. In one case it was possible that the IC also contained IgM.

CHAPTER . 7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

Crohn's disease (CD) presents several features which suggest participation of the immune response in its pathogenesis and aetiology. One of these is the finding of immune complexes (IC) in the sera of some patients with CD (Jewell and MacLennan, 1973; Doe, Booth and Brown, 1973; Hodgson, Potter and Jewell, 1977a; Hodgson, Potter and Jewell, 1977c; Fiasse, 1978). using a variety of methods. It has been suggested that ICs could be responsible for the extragastrointestinal manifestations often seen in this disease, i.e. uveitis, arthritis, vasculitis, liver disease and erythema nodosum (Hodgson, Potter and Jewell, 1977a). ICs are known to fix complement (C') (Mayer, 1973) and, as C' is one of the most important mediators of humoral immunity, the purpose of this thesis was to examine components of the C' system in relation to ICs in CD.

The activity of the C' system in sera of patients with CD was examined by CH_{50} assay (Chapter III). CH_{50} levels were found to be normal in contrast to CH_{50} levels in rheumatoid arthritis (RA) and ankylosing spondylitis (AS) which were markedly reduced. RA and AS are thought to be IC-mediated diseases (Norberg, 1974; Sturrock et al 1978). The finding of normal CH_{50} levels in sera from patients with CD initially indicates that there is no apparent disturbance of C' activity in this disease. However, estimation of CH_{50} levels in sera depends on many factors. CH_{50} assay is a measure of the activity of the whole C' sequence, from C1 to C9 (Takada, Imamura and Takada, 1979) via the classical pathway. It does not take into account individual C' components of the pathway. C' consumption may be compensated for by increased synthesis of one or more C' components. C3 plays a key role in activation of the

C' system by both the classical and alternative pathways. C4 is involved in the early stages of classical pathway activation. The serum concentrations of these two C' components were measured in patients with CD and were found to be significantly raised (Chapter III). Again, this contrasted to reduced levels found in sera from patients with AS and in synovial fluids from patients with RA. Serum levels of C3 have been reported to be raised in patients with RA (Versey, 1973). Some workers have found raised serum C3 and C4 concentrations in CD (Ward and Eastwood, 1974; Hodgson, Potter and Jewell, 1977b) whilst others have reported normal levels (Teisberg and Baklien, 1974; Teisberg and Gjone, 1975). Raised levels of serum C3 and C4 with normal CH_{50} levels requires explanation. Takada et al (1979) reported a positive correlation between CH_{50} and C3 and a weak correlation between CH_{50} and C4 in normal subjects. Serum concentration of a protein such as C3 and C4, reflects a balance between synthesis and catabolic rates, plasma volume and extravascular distribution (Hodgson, Potter and Jewell, 1977c). An increased serum concentration could be due to any of these factors. Kinetic studies of Hodgson, Potter and Jewell (1977c) have shown that both the fractional catabolic rate and rate of synthesis of C3 are increased in CD. This implies that C' activation is occurring. This will be discussed later (see page 135), but it does not discount that a raised level may result from an imbalance between synthesis and catabolic rates. Finally the method of measurement of C3 and C4 levels may give rise to apparently high serum levels. The method of measurement of concentration by single radial immunodiffusion (Chapter II) depends on the rate at which a protein diffuses across a gel. Inactivation products of C3 and C4 are smaller molecules and would have a faster rate of diffusion in a

gel thus giving an apparently higher serum concentration (Hodgson, Potter and Jewell, 1977b). This was thought to be unlikely, however, because no relationship was found between C3 levels and evidence of C3 inactivation (C3i) products (Chapter V). C3 levels were significantly raised whether C3i products could be demonstrated or not. C4i products were not measured but presuming C4 inactivation precedes C3 inactivation (classical pathway activation of C') then C4 levels were less likely to be falsely elevated since C4 levels were actually no higher than control values in those sera that showed C3 conversion. A positive correlation was seen between C3 and C4 concentrations indicating that activation was via the classical pathway.

Because ICs are known to activate the C' system. this fact can be utilized in their detection by anticomplementary activity assay. There are many methods of IC detection. No one method will detect all types of IC. ACA can be a useful method because it detects ICs that contain IgG and IgM by activation of C' via the classical pathway and possibly ICs that contain IgA, via the alternative pathway of C' activation (Neilsen and Svehag, 1976). ACA has been shown to detect ICs of small size, 11s, (Hodgson, Potter and Jewell, 1977a). In view of the fact that animal experiments have shown that large ICs are rapidly cleared from the circulation by the reticulo-endothelial system (Mannik, Haarkenstad and Arend, 1974) a method of detecting small ICs is desirable. The presence of small ICs may indicate that larger complexes are also being formed. The method of ACA assay used is cheap, easy, sensitive, reproducible and requires very small amounts of serum (25 μ l.). False positive results due to aggregated IgG can be avoided by careful handling of sera and heat inactivation procedures (Johnson,

Mowbray and Porter, 1973; Neilsen and Svehag, 1976; Hodgson; Potter and Jewell, 1977a).

Raised ACA found in 32.2% of sera from patients with CD (Chapter III) confirms the findings of Hodgson, Potter and Jewell (1977a) and is in agreement with the demonstration of ICs in the sera of a proportion of patients with CD by other methods (Doe, Booth and Brown, 1973; Jewell and MacLennan, 1973). Evidence of ICs was also found in all the sera tested from patients with AS and 55% of SF from patients with RA, using the method of ACA assay.

Gel filtration of ACA positive sera from patients with CD (Chapter VI) showed that ACA was confined to fraction pools of molecular (MW) range $10-2 \times 10^5$ daltons. This is greater than the M.W. of monomeric IgG (1.6×10^5 daltons) and IgA (1.7×10^5 daltons) but not IgM (9×10^5 daltons); IgG and IgA detected in these fractions were thought to be part of ICs or aggregates. IgG was unlikely to be aggregated since the IgG found in the high M.W. fraction was a small percentage of the total and it has been shown that for aggregates to bind C' they must be present in a high concentration (Neilsen and Svehag, 1976). IgA detected in high M.W. fractions may have been in the form of aggregates especially when the concentrations found in these fractions represented a high percentage of the total IgA (Chapter VI, patient JR). It was concluded that ACA found in serum of patients with CD was due to the presence of ICs containing IgG and possibly IgA.

Estimation of CH_{50} , ACA, C3 and C4 levels in sera gives a static picture of the involvement of C' in the disease process of patients with CD. It was hoped to gain a more dynamic view of C'

activation from the demonstration of C3i products in sera of patients with CD. C3i products were demonstrated in 32.9% of samples. This percentage is lower than the figures of 52.4% and 51.7% reported by Teisberg (1975b) and Teisberg and Gjone (1975), respectively. The discrepancy may be partly explained by the fact that there is much controversy over methodology. Since no evidence of C3 conversion was found in control subjects demonstration of C3i products in serum or plasma from patients with CD was taken as evidence of in vivo activation of the C' system. Activation of the C' system was thought to be caused by circulating ICs because their presence, as detected by ACA assay, could be correlated to C3 activation. Serum C3 levels were elevated whether C3i products were demonstrated or not, suggesting compensatory increase in synthesis. Kinetic studies of C3 metabolism in patients with CD (Hodgson, Potter and Jewell, 1977c) have shown that C3 synthesis and catabolism are increased. Serum C4 was found to be raised when no C3 activation was demonstrated but normal when C3i products were present. This suggests consumption of C4 during C' activation which was not compensated for by increased synthesis. Kinetic studies of C4 metabolism might provide evidence to substantiate this hypothesis.

CD is a chronic condition following an unpredictable course of quiescent periods punctuated by acute exacerbations. Drug therapy is often required, mainly the use of corticosteroids usually prednisolone, and immunosuppressants, usually azathioprine (AZA). These types of drugs might be expected to have an effect on immunological activity, as might length of history of disease and disease activity. The relationship of these parameters to C' and IC levels was examined in Chapter IV. No differences were

found that could be attributed to length of disease process. Other workers have also failed to find any correlation with disease history (Jewell and MacLennan, 1973; Hodgson, Potter and Jewell, 1977a). It appears that once the disease is established the underlying immunological process is continuous. Steroid therapy alone or in conjunction with AZA does seem to be effective in the treatment of CD (Willoughby et al, 1971; Rosenberg et al, 1976). although its use has recently been criticized. (Smith et al, 1978). However, no differences were seen in C' and IC determination whether the subjects were receiving steroid therapy or not. This confirms other reports of a lack of effect of steroid therapy on the presence of ICs (Jewell and MacLennan, 1973; Hodgson, Potter and Jewell, 1977a; Hodgson, Potter and Jewell, 1977b). The mechanism of action of steroids and immunosuppressants remains largely unknown. The beneficial effects of corticosteroids presumably include the anti-inflammatory actions, which may involve inhibiting the release of plasma kinins, a reduction of capillary permeability and the ability to make cell lysosomes more resistant to agents that cause the release of lysosomal enzymes (Kraft and Kirsner, 1971). The action of immunosuppressants, which may also apply to corticosteroids, may involve the reduction of the effects of antibody-antigen interactions at or near cell surfaces. The drugs may have a local effect on cell populations in the gut but no effect on circulating ICs.

Disease activity could not be correlated with the presence of ICs in the patients with CD studied although other authors have reported increases in circulating ICs and C' components with increase in severity of disease (Jewell and MacLennan, 1973; Teisberg and Gjone, 1975; Hodgson, Potter and Jewell, 1977a;

Hodgson, Potter and Jewell, 1977b). C3 and C4 levels were higher in patients with active disease, but not significantly so. The distinction between active and quiescent disease was made subjectively according to clinical criteria. Other workers have employed more detailed discriminatory criteria for assessment of disease activity. This was not feasible under the conditions of this project. This could account for the lack of differences in immunological activity between patients in active and quiescent phases of the disease. However, the fact that ICs were detected and elevated serum levels of C3 and C4 were found in patients with quiescent disease lays emphasis on the suggestion that C' activation must be involved in the immunopathology of the disease (Ward and Eastwood, 1974).

SUGGESTIONS FOR FUTURE WORK

This investigation has shown consistently elevated levels of C' components C3 and C4 in the serum of patients with CD, despite the finding of activation of the C' system from ACA assay and the demonstration of circulating C3i products. From the experiments performed in this investigation it was concluded that ACA resulted from the fixation of C' by IgG- and possibly IgA-containing complexes with a MW of between $>10^6$ and 2×10^5 daltons. It would be of value to pursue methods of IC detection that are independent of C' fixation. Preliminary attempts were made to do this by the inhibition of Fc-rosette formation by sera from patients with CD. The method has been described by Morito et al (1976) and shown to detect ICs in the sera of patients with SLE. Secondly, in order to elucidate the cause of raised C' levels it would be useful to examine the metabolism of C3 and C4 by kinetic studies.

Because the site of the pathological lesion is in the bowel, it may be more relevant to direct future investigations to examination of immunological parameters local to the site of gut injury, using biopsy and resected material. Immuno-fluorescent studies have shown evidence of ICs in the bowel mucosa of patients with UC (Ballard and Shiner, 1974). Similar studies may provide information towards elucidation of the mechanisms of the aetiology of CD.

Because of the known modifying effects of prostaglandins on the immune system and because of the involvement of prostaglandins in UC it would be useful to investigate any role they may have in the initiation or periodic resolution of CD through their inhibition of cytotoxic reactions. Knowledge of the modifying action of prostaglandins could lead to a rational approach to

therapy in the disease by the use of agents which act in a similar (e.g. choleratoxin, β -adrenergic agents, histamine, phosphodiesterase inhibitors) or opposite (α -adrenergic agents) fashion on cyclic AMP levels, as this agent is believed to function as a second messenger following prostaglandin stimulation. Obeying the dictats of the Yin Yang hypothesis it would be logical to examine whether agents which increase cyclic GMP (such as acetyl choline and carbamyl choline) has an opposite influence on cytotoxic reactions to those which increase cyclic AMP levels.

Finally, investigations should concentrate on newly diagnosed patients in whom immunological damage is probably in its most active phase.

APPENDIX

APPENDIX

I. FORMULAE

Barbitone buffer, pH 8.6, ionic strength 0.1

barbitone sodium 9 gm.

0.1N hydrochloric acid 65 ml.

sodium azide 0.5 gm.

distilled water 935 ml.

Phosphate buffered saline (PBS) pH 7.35 ± 0.05

sodium chloride 32 gm.

potassium chloride 0.8 gm.

disodium hydrogen phosphate 5.1 gm.

potassium dihydrogen phosphate 0.8 gm.

distilled water 1 litre.

Coomassie Brilliant Blue stain

Coomassie Brilliant Blue 5 gm.

methanol 450 ml.

glacial acetic acid 10 ml.

distilled water 450 ml.

Destaining solution

methanol 450 ml.

glacial acetic acid 10 ml.

distilled water 450 ml.

Barbitone buffer, pH 8.6, ionic strength 0.1

Stock barbitone sodium 20.6 gm.

barbitone 4.0 gm.

sodium azide 1.0 gm.

distilled water 1 litre.

working strength Stock diluted 1 in 5 with distilled water
to give ionic strength 0.02.

Borate buffer, pH 8.6, ionic strength 0.05

boric acid 6.7 gm.

disodium tetraborate 13.4 gm.

sodium azide 0.5 gm.

distilled water 1 litre.

10/150 buffer, pH 7.4.

150 mM sodium chloride in 10 mM phosphate

II. MANUFACTURERS AND SUPPLIERS

Wellcome Reagents Ltd.,
Wellcome Research Laboratories,
Beckenham,
Kent.

1. Horse anti-sheep red blood cell haemolytic antiserum
Cat. No. VD 16;
2. Freeze-dried guinea-pig complement 2 ml.
Cat. No. CT 01;
3. Well cutter;
4. Antisera to human IgG Cat. No. IP 26
IgA Cat. No. IP 25
IgM Cat. No. IP 27;

Oxoid Division of Oxo Ltd.,
London.
EC4

1. Sheep red blood cells in Alsevers solution
Cat. No. SR 53;
2. Complement fixation test buffer tablets
Cat. No. BR 16;
3. Ionagar No. 2
Cat. No. L 28;
4. I.D. agar tablets
Cat. No. BR 27;

Hoechst U.K.,
Hounslow,
Middlesex.

1. Antiserum to complement component C3 Cat. No. OTEA 04/05;
C4 Cat. No. OTNC 04/05;
2. Human standard serum
Cat. No. ORDT 02/03;
3. Human standards for IgG Cat. No. OTRA 06/07;
IgA Cat. No. OTRB 06/07;
IgM Cat. No. OTRC 06/07.

Flow Laboratories Ltd. (Titretek),
Irvine,
Scotland.

1. Microtitre plates, 8x12 U-shaped wells Cat. No. 76-311-05;
+ lids Cat. No. 76-406-05;
+ sealers Cat. No. 76-401-05;
2. Tuilip loop microdilutors, 25 μ l.
Cat. No. 76-742-02;
3. Disposable plastic pipettes, 25 μ l
Cat. No. 77-021-06.

Pharmacia Fine Chemicals A.B.,
Uppsala,
Sweden.

1. Sepharose 6B
Cat. No. 17-0140-01;
2. 100x2.5 cm. column, SR 25/100
Cat. No. 19-0880-01.

British Drug Houses Ltd.,

Poole,

Dorset.

1. Agarose
Cat. No. 33006;
2. Coomassie Brilliant Blue
Cat. No. 15211 2A.

Shandon Southern Instruments Ltd.,

Camberley,

Surrey.

1. Electrophoresis tank with cooling plate, U77
Cat. No. SAE 3225;
2. Power Pack, V500/150
Cat. No. SAE 2770;
3. Horizontal table and spirit level;
4. Gel cutter and cutting blade.

V.A. Howe and Co. Ltd.,

London. SW6.

1. Hamilton microsyringe
Cat. No. 705;
2. Adaptor
Cat. No. 83700.

Amicon Corporation,
High Wycombe,
Bucks.

1. Amicon filter
Cat. No. 202;
2. Diaflo membrane
Cat. No. PM 10.

Kodak U.K.,
Kirkby,
Liverpool.

1. 5x5 cm. glass plates

LKB Biochrom Ltd.,
Cambridge.

1. Combi coldrac 5201
2. Biocal chart recorder
3. Bromma 7000 Ultrorac fraction collector.

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